ImaGene™

User Manual

Version 5.5
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Introduction to ImaGene

ImaGene is BioDiscovery's solution for a fast and accurate microarray image analysis software system. With ImaGene, researchers can generate quantified data within seconds or minutes while at the same time generating extensive quality control information.

ImaGene performs microarray image analysis for users. Microarray image analysis is used to quantify the relative expression levels as exist within a microarray scan. As such, the role of ImaGene within the microarray process is post hybridization and scanning. The subsequent results of ImaGene are quantified expression values that are saved to a text file. These results can then be used in a data analysis package, such as BioDiscovery's GeneSight, for further analysis.

Quick Start Guide

ImaGene is designed to be the fasted and easiest software solution for quantification of microarray images. The description below outlines the essential steps to required to setup ImaGene For additional information, please view the remaining sections of this user manual.

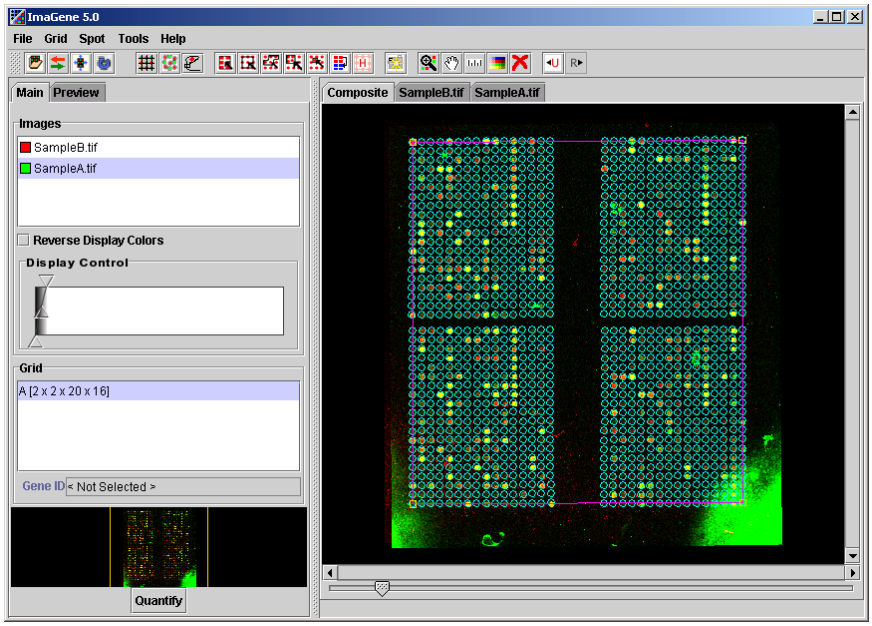
To setup ImaGene:

1. Install ImaGene.
2. Submit registration information and request a license from BioDiscovery.
3. Start ImaGene.
4. Load an image
5. Establish Settings
6. Perform grid placement
7. Quantify the Image.

Part 1: Lab Users Guide

1.1 ImaGene Main Window Overview

The ImaGene Main window is the primary program interface. It serves as the focal point of all work within ImaGene. You use this window to, among other things, load images, place grids, produce quantified data and review results. This chapter identifies the components of this window and explains how each area fits into the array analysis and data extraction process.



Menu Bar - Located along the top of the window. Click on one of the menus (*File*, *Grid*, *Spot*, *Tools*, or *Help*) to view the program commands available on that menu. The following options are available from the Menu Bar.

- File
  - Load Images – Allows selection of images to be loaded from the file system.
  - Remove Selected Image – The image which is selected, or highlighted, from within the images panel is removed, or unloaded, from analysis. Multiple images can be removed by highlighting several images then selecting Remove Selected Images.
  - Review Results – Allows selection of a snapshot, or sst file, which will allow for the results of previous analysis to be reviewed or

reexamined. Simply browse to and select the desired sst file and ImaGene will load previously created settings, data, segmentation and flagging information.
    - Batch Editor – Allows creation of batches to automatically process data. Selecting this option will open the Batch Editor Window allowing for entries to be added to a batch.
    - Save Display Image – Allows a 24-bit tiff image of the composite overlay to be created and saved to the file system. The image can be useful in post processing or generation of publication.
    - Settings – The Settings option opens the ImaGene Parameter Settings Window. This window and the tabs within the window contain all the parameters within ImaGene for Quality Measures, Auto Flagging, Spot Finding and Alert Logging.
    - Exit – Closes the ImaGene program.
- Grid
    - Load Grid - Displays the Load Grid dialog box. Use this interface to select and open a previously saved grid (.grd) file.
    - Save Grid - Displays the Save Grid dialog box. Use this interface to save a grid.
    - Clear Grid - Removes all displayed grids from the Image Display Panel.
    - Load Gene IDs - Displays the Load Gene ID File dialog box. Use this interface to select and open a gene ID (.txt) file.
    - Clear Gene Ids - Displays the Confirm Deletion dialog box. Use this interface to verify that you want to remove the displayed gene ID information.
    - Load Template - Displays the Load Template dialog box. Use this interface to select and open a template (.tpl) file. A template is a grid file, which contains gene ID information.
    - Save Template - Displays the Save Template dialog box. Use this interface to save a template.
- Spot
    - Adjust Metagrid – Allows you to select and move an entire metagrid.
    - Adjust Subgrid – Allows you to select and move an individual portion of the metagrid (subgrid).
    - Adjust Spot – Allows you to select and move one spot in a metagrid.
    - Lasso Adjust – Allows you to select and move a specific (free form) area of the metagrid.
    - Rectangle Adjust – Allows you to select and move a specific rectangular area of the metagrid.
    - Auto Adjust Spots – Tells ImaGene to automatically adjust each spot to better align them with the corresponding image.

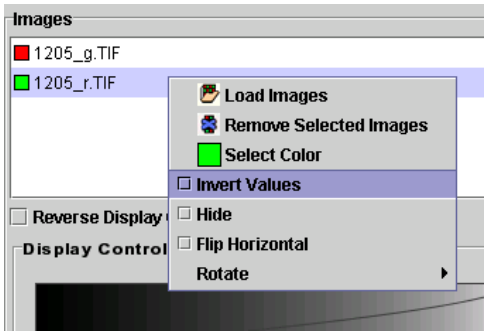
        - Wrangle - Enforces new local spot flexibility parameters. Essentially, reduces the distance used in spot finding without requiring spot finding to be reapplied.
- Tools Menu
    - Zoom – Turns the cursor into a magnifying glass. Use this tool to adjust the on-screen display size of images.
    - Scroll – Turns the cursor into a hand. Use this tool to scroll all images and grids at the same time.
    - Undo – Cancels the last executed command. For example, if you moved a spot, you could select Tools > Undo to cancel this action and, in effect, deselect the spot.
    - Redo – Restores the last canceled command. For example, if you used the Undo command to cancel a spot movement, you could select Tools > Redo to move the spot again.
    - Translate Images – Turns the cursor into a cross with arrows. Use this tool to move selected images.
    - Rotate Images –Turns the cursor into a circle. Use this tool to move selected images in a circular path based on a manually set anchor point in the image.
    - Ruler – Turns the cursor into a small ruler. Use this tool to measure the size of spots or distances between them.
    - Image Intensities – Turns the cursor into a small light bulb. Use this tool to measure the intensity of spots.
    - Tag Spots – Turns the cursor into the letter X. Use this tool to manually flag spots. Right click with this tool to see a list of available flagging options.
- Help
    - ImaGene Help - Displays the ImaGene Online Help documentation.
    - Wizard On/Off – Turns on and off the ImaGene Wizard. The wizard provides guidance on the proper steps to be performed within ImaGene.
    - Change Skins – launches a dialog for choosing between different skins for ImaGene interface (using skinlf). When a specific skin is selected, the change take effect next time you launch ImaGene.
    - Auto-Update – This option allows you to automatically update ImaGene package with the newest version available for download from BioDiscovery website. You can also set a frequency for automatic update check at ImaGene start-up.
    - Support Center – launches Web Browser with customer support page for ImaGene. Through that web-site you can access latest technical documentation, request a new feature, obtain sample images or templates etc.

- About ImaGene - Displays the About ImaGene dialog box. This interface contains information (license number, mode, etc.) about your copy of ImaGene.

Toolbar- Located directly beneath the menu bar. This region is composed of multiple buttons that provide a one-click method for executing program commands.

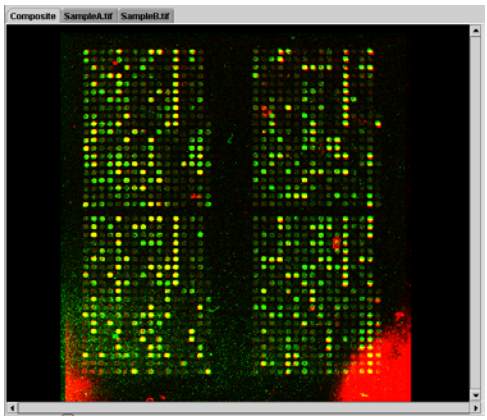
Context Menu – Context Menus are created by right clicking on the various elements within the ImaGene graphical user interface (GUI). Not all elements support context menus. Elements which do support this feature are listed below:

- Images Panel
- Grid Panel
- Image Display Panel
- Gene ID Selector
- Flagging Tool

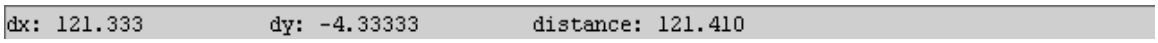


Control Tabs – Located to the left of the window. The control tabs provide information immediately relevant to the images being quantified. Please see section 1.2 for additional information on the control tabs.

Image Display Panel – Located to the right of the window. This panel displays loaded images and is where grid placement takes place.



Status Bar – Located at the bottom of the window. The status bar provides feedback to the user regarding loading and handling of images. Also, other tools such as the Ruler and Image Intensity display their information here.

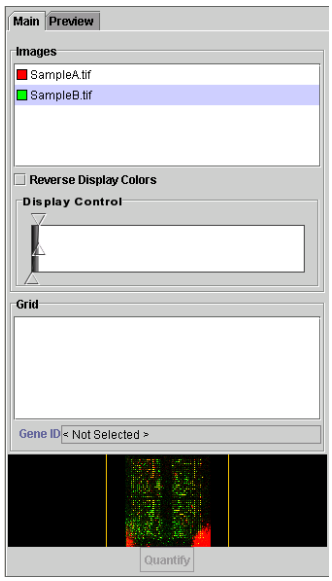


1.2 Control Tabs

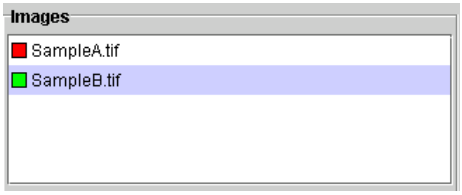
Located along the left of the main ImaGene window are two tabs containing essential information about the analysis.

1.2.1 Main Tab

The Main Tab is used to perform the essential steps of microarray image analysis, loading the image(s) to be processed and placing a grid on the image and its corresponding structure. The following Panels are contained within the Main Tab.

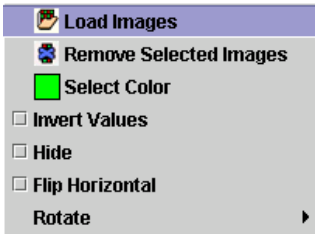


Images Panel
The images panel displays the names of images currently loaded within ImaGene. Each loaded image is listed here as well as its corresponding Composite color. The Composite Color is the color the image is displayed as when seen within the Composite Tab of the Main Image Panel.



The Images Panel fully supports context menus and as a result provides the following menu choices:

- Load Images – Allows browsing to and selection of images from the file system.
- Remove Selected Images - The images which are selected, or highlighted, from within the images panel are removed, or unloaded, from analysis.
- Select Color – Select the color to be used for the select image when seen under the Composite Tab. The default color for the first two images are red and green; however, these can be changed to any color desired. Changing the colors here in no way affects the resulting quantified values.
- Invert Values – This will invert all pixel intensities within the selected image. When the image is loaded, ImaGene automatically determines which end of the grayscale spectrum is the high value. This information is typically available within the image file itself. However, in rare cases, this information is not present within the file and as a result, ImaGene requires this information to be manually set. Should you determine the expected values for the signal and the background measurement to be opposite to what is expected, selecting Invert Values will solve the problem.
- Hide – The selected image will no longer be visible under the Composite Tab. While not visible, if quantified, data will be generated for the image.
- Rotate – Rotates the image 90, 180 or 270 degrees around the top, left of the image.



How to Load an Image(s)
The following steps describe the process of loading Images within ImaGene:

1. From the menu bar, select File followed by Load Images. Alternatively, click the first icon on the toolbar or right click on the Images Panel and select Load Images.
2. From the Load Images dialog that appears, browse to and select the desired file. Multiple files may be selected by holding the <shift> or <ctr> keys and left clicking on the image name.

Note: ImaGene supports the following image file formats:

- *Tiff – The file extension is tif*
- *MD Gel – The file extension is gel*
- *Fuji Bas – This is a two file format with one file ending in inf and the other ending in either img or bas.*

Reverse Display Colors - Mark this check box to reverse the displayed colors of all open images. Activating this feature has no affect on the Composite tab. Changing the appearance of an image does not affect the pixel intensity of your original data. This tool just makes it easier to see dim spots in an image.

Display Control

The Display Control allows adjustment of how the images are displayed. By moving the triangle located at the top and bottom the display control, the image can be lighted and darkened. The tool is designed solely to enhance viewing of the image and does not affect the quantified values generated by ImaGene. When adjustments are made to the Display Control, these are applied only to the Image(s) selected within the Images Panel.

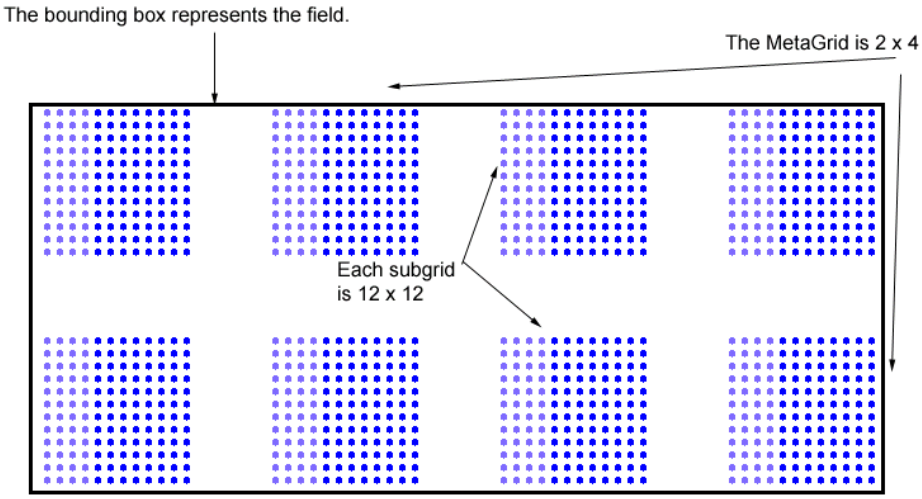
The specific elements of the Display Control are and perform the following:

- Top Triangle – This sets the minimum intensity to be displayed.
- Middle Triangle – Allows the rate of change of pixel intensities to be specified by changing the curvature of display curve.
- Bottom Triangle – This sets the maximum intensity to be displayed.

Grid Panel

The Grid Panel lists all fields that have been created and placed on the image. Typically, only a single field will be required; however, depending on the design of the array being quantified, several fields may be required. As a new field is created, it is added to the list.

Before explaining how and why to created a Field, let us explore some of the definitions and structures of a field. A field is the largest design element within a slide. A field typically consists of the arraying done by a single print head on the slide. For example, if the arrayer has a print head with 8 pins in a 2 x 4 configuration, the region of the slide containing the resulting printing is a field. The metagrid in this example would be 2 x 4 as the resulting printing would generate 2 rows by 4 columns of subgrids. The subgrid is not defined here, but would be whatever rows and columns of spots are printed by a single pin, 12x12 for example. The accompanying diagram demonstrates the relationship between the three levels of structure.



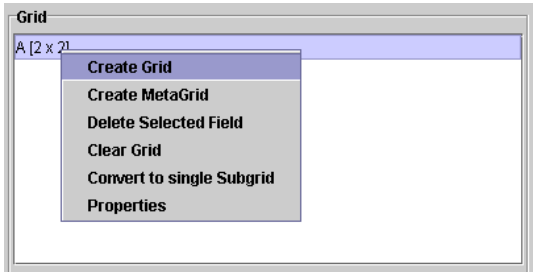
The Grid Panel like other components of ImaGene fully supports context sensitive menus. Right clicking on the Grid Panel provides the following options:

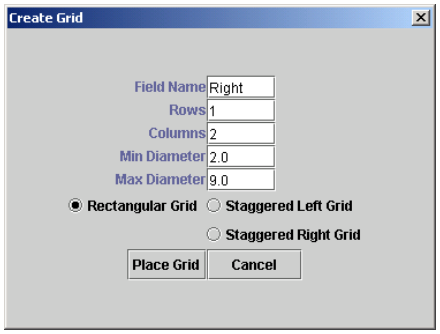
- Create Grid – The option launched the Grid Creation Window where the essential information about the design of the grid must be entered. Remember that within ImaGene the grid is the most elemental structure, followed by the metered and finally field. The following parameters must be specified:

- Field Name – Specify the name of the field here. The name may be any name desired. By default ImaGene provides the letter 'A' to the first fields, 'B' to the next field and so on. If a gene ID file is being used in conjunction the image to provide gene names or accession numbers within the text output file of ImaGene, the fields name, if used, must match between what is specified here and that is contained within the Gene ID file. For example, within the gene ID file, if I have a column where I specify the field and that field has the name "Top", then I must enter "Top" for the field name within ImaGene. If the name does not match, then ImaGene with NOT use the gene name within the data output file.
- Rows – The number of rows of spots contained within the grid. Typically, this is simply counted visually.
- Columns – The number of columns of spots contained within the grid. Typically, this is simply counted visually.
- Min Diameter – This specifies the minimum expected diameter of the spot and is measured in pixels. The size of the spot can best be determined by use of the Ruler Tool. Due to the variety of array types and the variability of individual arrays there is no set procedure for determining the minimum diameter here. The most common rule of thumb is to specify the size of approximately the 10 % of the smallest spots of the array. Depending on the type of array, if the spots are highly uniform, then the minimum diameter specified here will be close or equal to the maximum diameter specified next.
- Max Diameter – Similar to the Min Diameter specified above, this parameter reflects the maximum anticipated spot size measured in pixels. Ts with the minimum diameter this value can be approximated by using the top 10% of large spots and measuring the sizes with the Ruler Tool.
- Spot Orientation – Spot Orientation reflects how each row is located relative to the row that preceded it. The selections here reflect how the row above the subsequent row is position. While rectangular is the most common, all the possible selected are:
  - Rectangular
  - Staggered Left Grid
  - Staggered Right rid

- Create MetaGrid – This selection allows subgrids to be used to form a metagrid structure. Before creating a metagrid, at least one subgrid must be created. To create a metagrid, first highlight the desired subgrid, then select "Create Metagrid". The Create MetaGrid Window appears and requires the following parameters to be specified.
  - Metarows – The number of rows of subgrids.
  - Metacolumns – The number of columns of subgrids
- Delete Selected Fields – The currently highlighted field will be removed.
- Clear Grid – All fields that have been created will be removed.

- Convert to single Subgrid – If a metagrid structure exists and has been used to "grid" the image, selecting this option will convert the metagrid structure to a simpler subgrid structure. For example, if we have a 2x2 meta grid with 15x10 subgrids, after selecting this conversion, the resulting subgrid size will be 30x10. During this conversion nothing changes except for the how the individual spot location are represented. This feature is option and may used if required to construct a multi-level metagrids for example.
- Properties – This option opens a window, which displays the parameters for the currently selected field. Only certain options can be changed while the remainder required the grid to be deleted then recreated a new. The following parameters are displayed within the properties window:
    - Field - The name of the field. This value may be changed.
    - Metarows – The number of metarows previously specified
    - Metacolumns – The number of metacolumns previously specified
    - Row s– The number of rows of spots within the subgrid that was previously specified.
    - Columns - The number of columns of spots within the subgrid that was previously specified.
    - Min Diameter – The minimum expected diameter of the spots to be used during spot finding. This value may be changed but the results will not be visible until spot finding has been performed again.
    - Max Diameter - The maximum expected diameter of the spots to be used during spot finding. This value may be changed but the results will not be visible until spot finding has been performed again.





How to create Grid

Perform the following steps to create a subgrid within ImaGene:

1. Load the desired images into ImaGene

2. Right click with the mouse on the Grid Panel and select Create Grid
3. Specify the parameters within the create grid window. If a Gene ID file is being used then the field name must match the name of the field within the gene ID file. The min and max diameter can be calculated with the Ruler Tool.
4. Click the Place Grid Button and click on the four corners of a subgrid. If you make a mistake, right click with the mouse to remove the last placement.

Perform the following steps to create a metagrid within ImaGene:

1. Click on and select the desired subgrid to use as a basis for the metagrid. This can be done by left clicking on the grid within the Grid Panel.
2. Right click and select Create MetaGrid from the menu.
3. In the Metagrid Parameters Window, specify the number of rows and columns of subgrids contained within the metagrid.
4. Click Place Metagrid and click on the top left spot in each of the corner subgrids. Typically, this process will require four clicks, but never more.

How to Save a Grid

Once a grid has been created the following steps will save the grid for later use.

1. From the menu bar select Grid followed by Save Grid.
2. In the Save as Dialog browse to the location where you wish to save the grid file.
3. Specify a file name. ImaGene will automatically add the .grd file extension to the end of the name.

How to Load a Grid

To load a previously created grid, perform the following steps:

1. From the menu bar select Grid followed by Load Grid.
2. From the Load Grid Dialog that appears, browse to and select the desired grid file.
3. Click on one of the following radio buttons to select a grid placement method:

a. Place Manually - Select this option to set the location and size of the saved grid yourself. This is useful when images have been scanned at different resolutions or the overall position of the array shifted between scans. To place the grid manually, left-click on the four corners of the entire array structure. The grid will then be resized and positioned based on this information.

b. Place in Saved Position - Select this option to place the grid in the identical position of the original grid. Use this option if the size and resolution of the images has not changed

4. Finally, click the Open Button to place the grid.

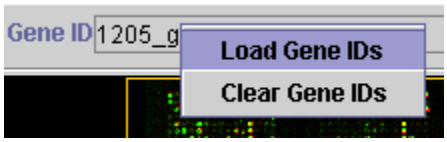
Gene ID

The Gene ID file allows you to track information about the genetic material spotted at each location within the array. This information will be saved along with the quantified values in the text output file and visualization tools.

If a Gene ID has been selected the name of the corresponding file is displayed here. The Gene ID also supports right click context menus with the following options:

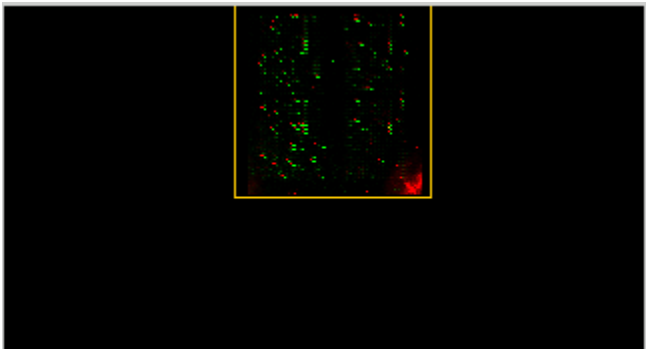
- Load Gene ID's – Selecting this option will open a window allowing for the selection of the gene ID file to be used. Likewise, you may also select "Grid" from the File Menu followed by "Load Gene IDs" to accomplish the same task. Note that while advantageous for a number of reasons, loading a Gene ID file is not mandatory. Please see the section of Gene IDs later within this manual for additional information on Gene IDs as well as appropriate file format.

- Clear Gene ID's – If a gene ID has already been selected, this option will remove the selected file from use. Likewise, you may also select "Grid" from the File Menu followed by "Clear Gene IDs" to accomplish the same task.



Template
You can load and save a template file that will contain both grid structure and corresponding gene IDs. For this purpose you can use BioDiscovery's serialized data format *.tpl, or you can import and export the template using several well-known formats.
GAL – tab-delimited text file containing location and structure of every subgrid (called "Block" within this format) and gene IDs.
GEML (v 1.0) – XML standard representing a "pattern" that can not fully describe a grid structure, but rather provides location and gene ID info for every spot.
MAGE-ML - the most complete XML format, imported/exported file will contain DesignElement_package and ArrayDesign_package of MAGE standard. This format can support multiple subgrids and metagrids.

Map View Panel
The Map View provides a comprehensive and unabridged view of the image while indicating exactly where within the image the primary image display panel is zoomed to. The Map View displays the entire image exactly as it appears along the main image display's composite view. Any corrections to contrast or rotation will likewise be visible within the Map Window. The Map View allows users with large arrays to more easily scan the image for proper gridding and segmentation.



The part of the image currently being displayed within the main image display is bound by a yellow rectangle within the Map View. You may also zoom using the Map View by left clicking and dragging with the mouse to select the desired region. To zoom completely out and display the entire image within the main image panel, double right click with the mouse.

Quantify

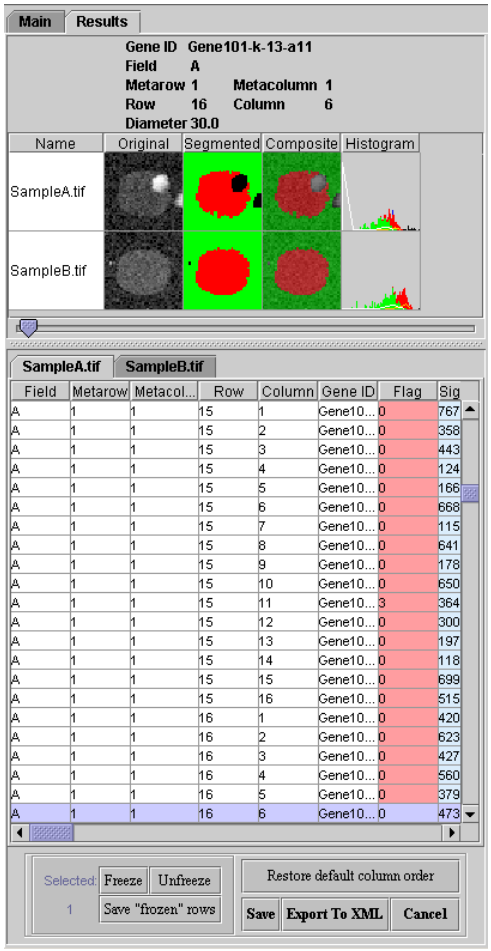
The act of quantification coverts the visual pixel intensities into numerical values to be used later in expression analysis. The Quantify Button starts this computation within ImaGene and should be performed after all other parameters have been set and the grid has been placed. The amount of time quantification takes is directly proportional to the speed of the computer hardware. Once quantification is completed, the Preview Tab becomes highlighted and the numerical values become visible.

Additionally, after quantification complete, an additional tab for each image becomes available over the main image panel. This tab is the segmentation tab and displays the segmentation, which has been performed across the entire image.

1.2.2 Preview Tab

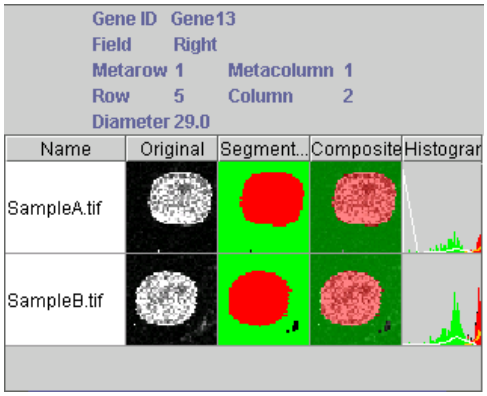
The Preview Tab displays information about the image both prior to and after quantification. Numerical values and segmentation can easily be viewed and reviewed to determine that optimum settings are established. The Preview Tab is divided into two primary parts:

- Segmentation Preview
- Quantification Preview


Main
Results
Gene ID Gene101-k-13-a11
Field A
Metarow 1 Metacolumn 1
Row 16 Column 6
Diameter 30.0
Name
Original
Segmented
Composite
Histogram
SampleA.tif
SampleB.tif
SampleA.tif
SampleB.tif
Field Metarow Metacol... Row Column Gene ID Flag Sig
A 1 1 15 1 Gene10... 0 767
A 1 1 15 2 Gene10... 0 358
A 1 1 15 3 Gene10... 0 443
A 1 1 15 4 Gene10... 0 124
A 1 1 15 5 Gene10... 0 166
A 1 1 15 6 Gene10... 0 668
A 1 1 15 7 Gene10... 0 115
A 1 1 15 8 Gene10... 0 641
A 1 1 15 9 Gene10... 0 178
A 1 1 15 10 Gene10... 0 650
A 1 1 15 11 Gene10... 3 364
A 1 1 15 12 Gene10... 0 300
A 1 1 15 13 Gene10... 0 197
A 1 1 15 14 Gene10... 0 118
A 1 1 15 15 Gene10... 0 699
A 1 1 15 16 Gene10... 0 515
A 1 1 16 1 Gene10... 0 420
A 1 1 16 2 Gene10... 0 623
A 1 1 16 3 Gene10... 0 427
A 1 1 16 4 Gene10... 0 560
A 1 1 16 5 Gene10... 0 379
A 1 1 16 6 Gene10... 0 473
Selected:
1
Freeze
Unfreeze
Save "frozen" rows
Restore default column order
Save
Export To XML
Cancel

Segmentation Preview

Use the Segmentation Preview to view the effects of current parameter settings on the segmentation both before and after quantifying the data. You select the spot to view and the corresponding information about the spot displays in the segmentation panel. At the same time selected spot can be seen on the image and two of the plots (Scatter plot and Box plot). This interface also supports dynamic analysis within ImaGene. You can adjust settings, such as the maximum and minimum signal values, and see the effects of these changes in real time in the dialog box.



The following textual spot information is available via the Segmentation Preview:

- Gene ID - Lists the corresponding information, typically name or accession, from the Gene ID file (if you imported a Gene ID file into ImaGene.
- Field - Lists the field that the selected spot belongs to. This name is specified when the grid was first constructed.
- Metarow - Identifies the row within the metagrid where the selected spot is located.
- Metacolumn - Identifies the column within the metagrid where the selected spot is located.
- Row - Identifies the row within the subgrid where the selected spot is located.
- Column - Identifies the column within the subgrid where this spot is located.
- Diameter - Lists the diameter, measured in pixels, of the selected spot. The diameter is determined during spot finding when various spot sizes are attempted.

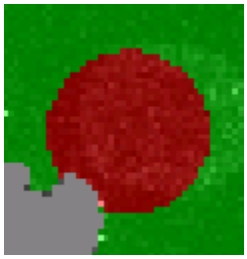
Note: Segmentation involves the partitioning of a microarray image into a set of regions
that convey a specified meaning. For microarrays, the purpose of segmentation is to
decompose a scanned optical image into regions that are meaningful in terms of spot
signal versus background.

The following visual spot information is available via the Segmentation Preview:

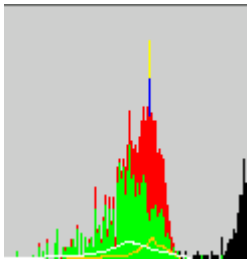
- Name - Lists the name of the source image file for the spot.
- Original - Displays the spot and its surrounding background without any segmentation information.

- Segmented - Displays an image of the segmentation, or pixel determination, that will be performed during quantification. The red pixels represent signal values and the green pixels represent background values. Black means the pixel is ignored.

- Composite - Displays an overlay between the *Original* and *Segmented* images. This image is *key* to determining if the best settings have been entered on the Measurements tab. Use this view to modify the settings until the desired signal and background values are included while removing contaminants.



- Histogram - Represents the distribution of pixels along the intensity scale for the spot. The y-axis (vertical) represents the number of pixels and the x-axis (horizontal) represents the range of intensities of pixels across the spot. The histogram is intended for a simple qualitative preview and should not be used for any form of data analysis. Within the histogram, colored vertical bars indicate specific values. The colors and their meaning are:
  - Blue – Signal Mean Value
  - Yellow – Signal Median Value
  - Cyan – Signal Mode Value



Under the Segmented, Composite, and Histogram Views color is used to indicate pixels to be included within the signal and background measurements. If a pixel is not color, then the pixel is being ignored and is not being used for calculation of either signal or background values.

The Preview Panel colors include:

- Green – Indicated the pixel is used in the background calculation
- Red – Indicated the pixel is used in the signal calculation
- No color (or black on histogram) – Indicated the pixel is used in neither calculation of signal nor background

Quantification Table

The Quantification Table displays the quantified numerical intensity values for each of the spots prior to being saved. All measurements previously selected to be quantified, including all quality measures are indicated here. Additional information regarding flagging and spot location information is provided.

Each section of the data, as presented by a grouping of columns, is color coded to facilitate easy review of the analysis. The following color codes describe the table:

- Red – Quality flag code
- Cyan – Quantified intensity values – mean, median, quality measures etc
- Purple – Spot location related information
- Yellow – Quality criteria failures and controls warnings

Gene ID	Flag	XCoord	Control	Background Mode
Gene34	0	282.0...		343.733333333...
Gene42	0	325.7...		71.8
Gene50	0	369.5...		47.2263157894...
Gene58	0	413.2...		55.5615763546...
Gene66	0	457.0...		66.2060301507...
Gene74	0	500.7...		41.796875
Gene82	0	544.5...		47.3885350318...
Gene90	0	588.2...		149.97
	0	632.0...		72.4493392070...
	0	675.7...		68.8995215311...
	0	719.5...		51.8888888888...
	0	763.2...		24.8266666666...
Gene3	0	107.2...		116.180602006...
Gene11	0	151.0...		103.349593495...

Note: By default these colors are grouped together, but you can drag and drop the column headers to change this order. Should the default order of the columns change, you can restore the columns to the default order by using "Restore default column order" button in the bottom of the table.

The default measurement order in the table is as follows.

- Field – Name of a field where the spot is located
- Metarow – Number of metarow in the metagrid where the spot is located
- Metacolumn – Number of metacolumn in the metagrid where the spot is located
- Row – Number of row in the subgrid where the spot is located
- Column – Number of column in the subgrid where the spot is located
- GeneID – Gene ID information for the spot
- Flag – Numeric code of the flag for the spot (0 – no flag, flag codes 1,…,7)
- Signal Mean – Pixel intensity averaged over the local signal region
- Background Mean – Pixel intensity averaged over the local background region
- Signal Median – Median pixel intensity computed over the local signal region
- Background Median – Median pixel intensity computed over the local background region
- Signal Mode – Mode pixel intensity computed over the local signal region (mode corresponds to the pick location in intensity distribution)
- Background Mode – Mode pixel intensity computed over the local background region
- Signal Area – Number of pixels in the local signal region
- Background Area – Number of pixels in the local background region
- Signal Total – Total pixel intensity summed over the local signal region
- Background Total – Total pixel intensity summed over the local background region
- Signal Stdev – Standard deviation of pixel intensities over the local signal region
- Background Stdev – Standard deviation of pixel intensities over the local background region
- Shape Regularity – First signal area of a spot is inscribed into a circle. Than number of non-signal pixels that fall within this circle is computed and divided by circle's area. This ratio is subtracted from 1 as is called "shape regularity"

- Ignored Area – Area of ignored regions directly neighboring ("touching") the signal area is computed
- Spot Area – Signal Area plus Ignored Area
- Ignored Median – Median pixel intensity computed over the local ignored region
- Area To Perimeter – This quality measure defines spot's circularity. Area of a spot is divided by a square of spot perimeter and multiplied by 4π . As a result, this measure ranges from 0 (highly non-circular shape) to 1 (a perfect circle)
- Open Perimeter – Computes the proportion of signal perimeter that touches the border of rectangular snip around the spot
- XCoord – X coordinate (in pixels) of grid circle corresponding to the spot
- YCoord – Y coordinate (in pixels) of grid circle corresponding to the spot
- Diameter – Diameter (in pixels) of grid circle corresponding to the spot
- Position Offset – Offset (in pixels) of the center of the grid circle from the expected position in the grid
- Offset X – X offset (in pixels) of the center of the grid circle from the expected position in the grid
- Offset Y – Y offset (in pixels) of the center of the grid circle from the expected position in the grid
- Expected X – X coordinate of expected position of the circle in the grid. Expected position in the grid is computed fitting least square lines to circle centers in every row and column
- Expected Y – Y coordinate of expected position of the circle in the grid. Expected position in the grid is computed fitting least square lines to circle centers in every row and column
- CM-X – X coordinate of the center of the mass of spot's signal region
- CM-Y – Y coordinate of the center of the mass of spot's signal region
- CM Offset – Offset (in pixels) of the spot's center of the mass from the expected position in the grid
- CM Offset-X – X offset (in pixels) of the spot's center of the mass from the expected position in the grid
- CM Offset-Y – Y offset (in pixels) of the spot's center of the mass from the expected position in the grid
- Min Diam – Diameter of the circle inscribed into the spot's signal region
- Max Diam – Diameter of the circle, the spot's signal region can be inscribed in
- Control – Name of a control type for current spot (no name means the spot is not a control spot)
- Failed Control – 0 if the control passed all tests, 1 if at least one of the tests failed
- Background Contamination Present – 0 if the spot passed background contamination test, 1 if it did not (see section 1.4)
- Signal Contamination Present - 0 if the spot passed signal contamination test, 1 if it did not (see section 1.4)
- Ignored % failed – 0 if the spot passed ignored percentage test, 1 if it did not (see section 1.4)
- Open Perimeter Failed – 0 if the spot open perimeter test, 1 if it did not (see section 1.4)

- Shape Regularity Failed – 0 if the spot passed shape regularity test, 1 if it did not (see section 1.4)
- Perim-To-Area – 0 if the spot passed perimeter-to-area test, 1 if it did not (see section 1.4)
- Offset failed – 0 if the spot passed offset test, 1 if it did not (see section 1.4)
- Empty spot – 1 if the spot was qualified as empty, 0 if it was not (see section 1.4)
- Negative spot – 1 if the spot was qualified as negative, 0 if it was not (see section 1.4)

Some of the measures can be excluded from or added to the table at any moment through "Measurements" panel of Settings dialog box.

Individual spots can be selected for review, either by selecting the row from within the Quantification Table or by selecting the spot within the image. If the spot is selected from the image, the Quantification table will automatically scroll to the proper location and the corresponding spot row will be highlighted. Notice also that the Segmentation Preview automatically updates and displays the segmentation information for the selected spot.

Save

Once the data has been quantified and you are satisfied with the results, the final step is to Save the data. ImaGene will save the data to common tab delimited test files, which can easily be opened in other programs such as Microsoft Excel or Notepad. ImaGene will save each image's data to a separate file and will automatically name the file based upon the name of the image.

Note: By default, ImaGene does not compute ratio values between images and save this information for a text file. To compute ratio information, please use GeneSight-Lite. Due to the large number of post image analysis options and to be as flexible as possible, BioDiscovery as separated all post image analysis processing out of its ImaGene Analysis. Basic mathematics, such as generating ratio value can be performed with GeneSight-Lite. If additional more advanced computations are required, BioDiscovery recommends GeneSight for full data analysis and visualization. However, there is an access to ratio data directly from ImaGene through Histogram plot under the "Plots" tab on Image Display Panel.

Export to XML

Before saving the data you also can export the measurements in GEML or MAGE-ML format.
GEML (v 1.0) format – GEML profile export, only four values per spot are available (signal/background value and standard deviation). User can choose between mean, median, mode for export as signal/background value. For more details go to http://www.rosettabio.com/products/conductor/geml/default.htm.
MAGE format (XML) – the most complete format, exported file will contain QuantificationType_package, BioAssay_package and BioAssayData_package of

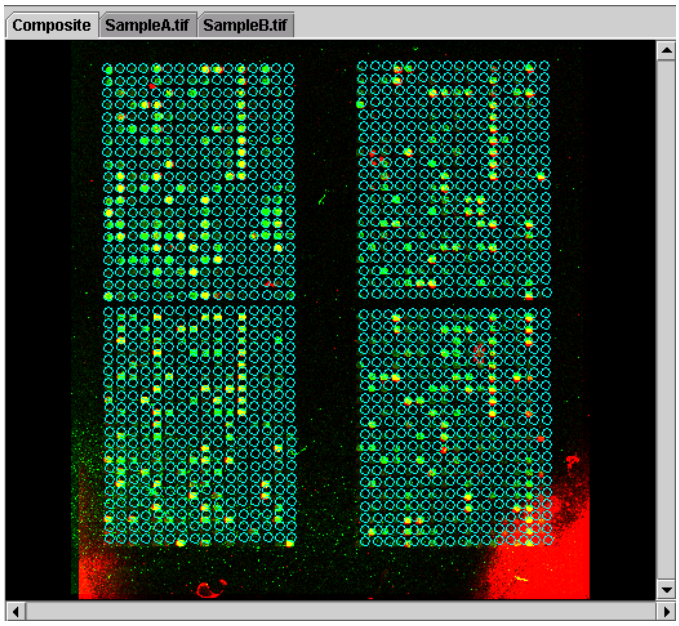
MAGE standard. All ImaGene measurements will be exported. For more details go to http://www.mged.org.
Note: once you export the data, the results table will not be closed, you can continue analyzing the data or save the results in usual format.

Cancel
Cancel clears the quantified data values without saving them. Once the quantification is cancelled, the image will need to be reprocessed before the data may be saved again. Upon selecting Cancel, you will be prompted to verify your action. Clicking yes will then clear the data. The Quantification table will no longer be visible.

Selection
Starting with 5.5 version of ImaGene, multiple spot selection is allowed. You can select several spots at a time pressing "Ctrl" or "Shift" keys when making selection in the results table. Indicator in the bottom of the table will show you how many spots are selected.
You can also "freeze" the selection using corresponding button. When "frozen", table rows will be highlighted in dark blue color and they will have flag "Selected" in the right-hand end of the table turned to "1". When you "freeze" the spots you can analyze them one by one by selecting only one row at a time. You can "unfreeze" the selection at any time and the spots will become highlighted with a usual selection color. This feature is useful if you selected the spots using one of the plots (selected regulated genes using Histogram for instance) and want to analyze their images, segmentations and quantifications results. You also can Save the frozen rows into .txt file.

1.3 Image Display Panel

This panel displays loaded images. A tab appears along the top of the panel for all currently loaded images. Click on a tab to display the corresponding image. The Composite tab displays a false color overlay for all loaded images. You can use this tab to overlay multiple images prior to analysis. The number of images which ImaGene can load is limited based upon the computer hardware specifications.



All image manipulation tools, such as zoom and rotation, can be applied within the image panel. Once zoomed into a region of the image, scroll bars become available along the sides of the panel.

Note: There is a Zoom slide bar located at the bottom of the panel. Move it to the left to zoom out of the image or move it to the right to zoom into an image. The Zoom slide bar is one of four tools available for zooming.

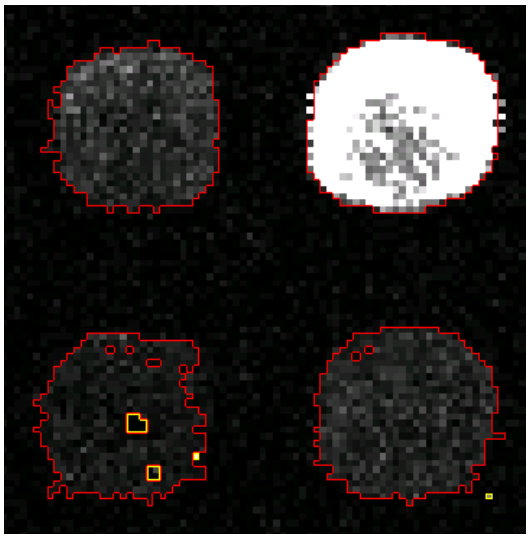
Segmentation Tabs

Once the image(s) have been quantified additional tabs, one for each image plus one for plots, will appear along the top of the image display panel. Some of these new tabs display the segmentation as it has been performed for the given image. From this view, you have the ability to see the macro view of the image and notice any large defects and the corresponding segmentation. Typical use of the segmentation tabs is for detailed post processing quality assurance analysis.

Unlike the Preview Panel, the Segmentation tabs use lines to indicate the segmentation's signal and background regions. All signal regions are surrounded by RED lines. All ignored regions, values not counted as signal or background, are surrounded by YELLOW lines. The remaining pixels within the image are all background regions.

The Segmentation Tab line colors:

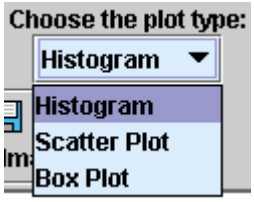
- Red – All pixels within the red lines are signal values
- Yellow – All pixels within the yellow lines are ignored pixels



Note: The description and use of colors between the Preview Panel and the segmentation tab does vary slightly. The segmentation tab includes the use of yellow to indicate ignored pixels where as the Preview Panel uses no coloring to indicate ignored regions. Also, the segmentation tab does not use a color to indicate the background region; however, within the Preview Panel, background values are indicated by a green color. The differences between the two displays is accounted by the fact that due to customer requests, the segmentation tab is designed to prevent eyestrain during extensive visual inspection.

Plots Tab

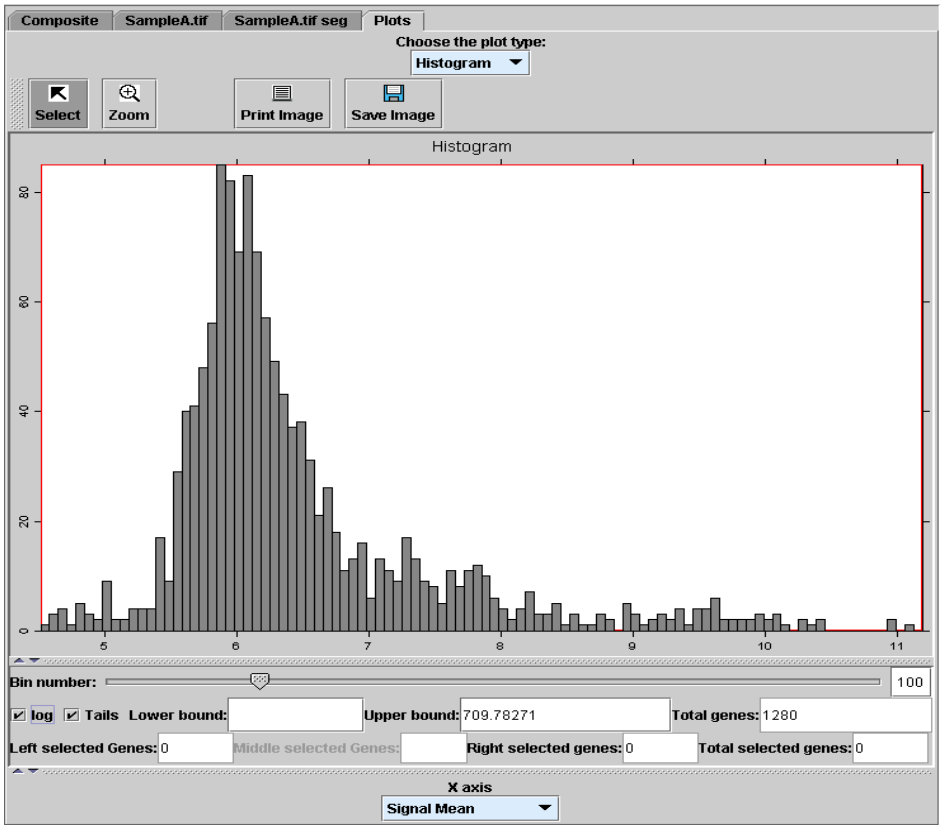
This tab appears also only when the image is quantified. It contains a set of useful data visualization tools that can help speed up the analysis process.



You can take virtually any measurement available from the results table and plot it in a manner corresponding to one of three tools: Histogram, Scatter Plot, Box Plot. Once you choose desirable type of visualization, choices of the measurement for X and Y axes become available. If Histogram is selected, only the measurement choice for X axis will be available.
Any plot can be printed or saved as an image file.

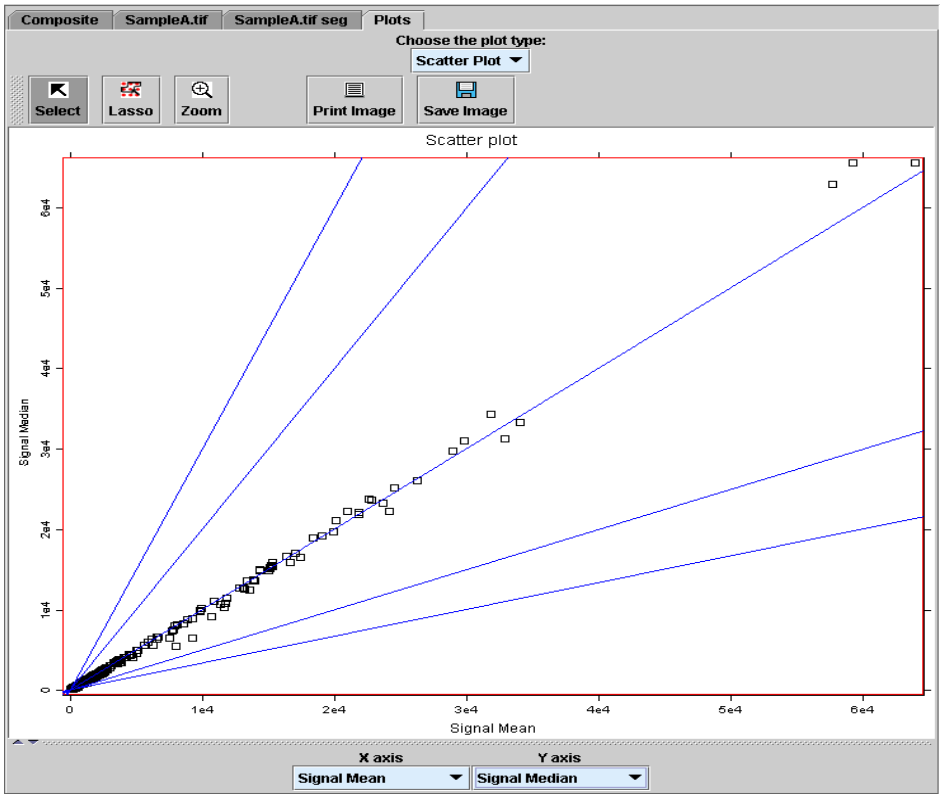
Change in measurement selection will be followed by update of the current plot.

- **Histogram.** As we mentioned previously, Histogram allows to choose only one measurement.



The histogram demonstrates distribution of the measurement values over their domain. One can make interval selection on the histogram and see how many genes fall into that selection. By changing selection of "Tails" check box you can select genes either inside the interval or outside. You can switch between untransformed measurement and log-transformed measurement using "log" check box. You can also change bin density, print histogram and save its image (this feature is especially useful for publications). Histogram provides not only ability to plot any of the measurements available in quantification table, but also gives you an access to such useful values as inter-channel signal ratios (including background corrected). Using these values in combination with "logarithmic" option you can obtain a histogram of a log-ratio (natural logarithm will be used). Selection of the tails of such distribution provides you a quick way to analyze up- and down-regulated genes.
Note: when selection is made on the histogram, same spots will be selected in measurements table and on the image, allowing you to analyze selected spots in full scale.

- **Scatter Plot.** Scatter plot offers visualization of one measurement plotted against any other.

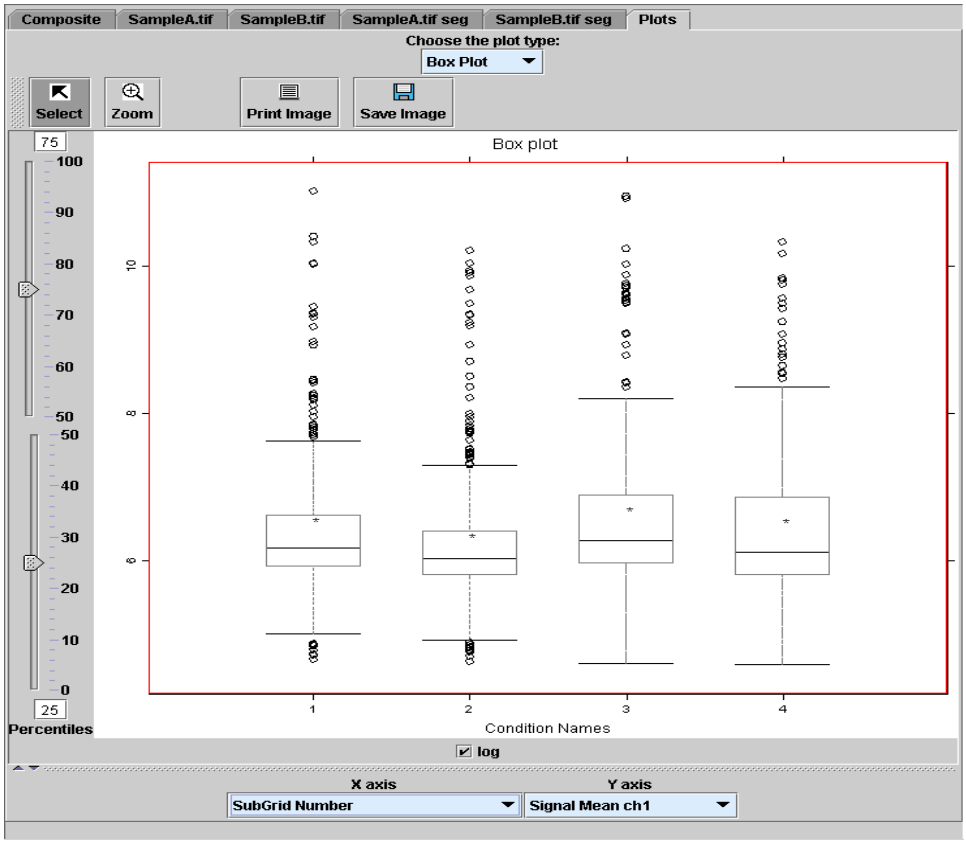


If multiple images are loaded, user can plot not only two measurements within one channel, but also two measurements belonging to different channels. For instance, plot of signal means can show the difference between fluorescence characteristics in two channels or even can give a rough idea about regulation of the genes. Selection of points on the scatter plot will result in selection of corresponding rows in the results table and highlighting of selected spots on the image.

Note: you can add to current selection by simply continuing to select other groups of spots. To start a new selection, right-click on the plot first to clear the previous selection.

- **Box Plot.** This type of plot allows you to visualize measurement's distribution between different categories of spots (belonging to different subgrids, different rows, having different flags etc). You choose between different categories for X axis and between different measurements for Y axis. The box on such graph visualizes lower and upper percentiles of distribution. These percentiles can be changed using scroll bars on the left hand side. Box plot also visualizes distribution outliers. Such plot can be useful when looking for abnormalities in the data due to special or other categorization.

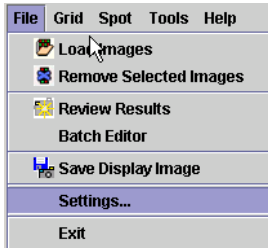
  Note: for more detailed description of Box plot refer to GeneSight manual provided with ImaGene.



Note: any selection that you make using one of the plots will be common for all other plots, results table and image tab. There is an exception, though; if you select a group of spots using Scatter plot, Box plot or Results table, the selection will not be visible on Histogram plot because of its specifics. But if you make selection using Histogram, it can be viewed by all other tools.

1.4 ImaGene Parameter Settings Window

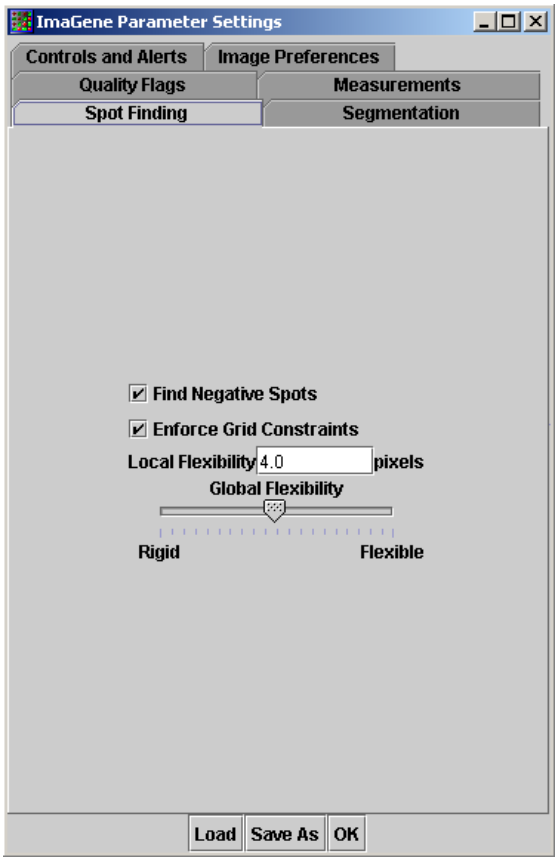
ImaGene encapsulates most of its parameters and settings within one common interface, the ImaGene Parameter Settings Window. Within this window are tabs, which control virtually all aspects of the array image analysis. From initial spot finding settings, to complicated auto reporting of alert values, the ImaGene Parameter Settings window provides complete user control. Selecting File, then Settings, may open the window.



The window is organized into several tabs which group related parameters together. The tabs available and their parameters include:

Spot Finding

Spot finding involves the localization of the array signal as printed on the array medium. Due the warping of the medium or potentially bent pins during printing, spots and sometimes even entire subgrids require spot finding in order to properly determine the location of the signal value. Depending on the type and characteristics of the array, if spot finding is not performed, the resulting quantification may be very dubious indeed.



The following parameters affect how ImaGene performs spot finding:

- Find Negative Spot – While not a problem for most arrays, negative spots can potentially cause problems for ImaGene spot finding. If you suspect that the image may contain negative spots, BioDiscovery recommends leaving this option checked. When Find Negative Spots is enabled, ImaGene will look for negative spots while also looking for regular spots.

  Note: If you suspect all the signal values within the image are negative due to specific scanner settings, then you may need to invert value for the images. Occasionally, the scanner software does not save information about which values are high and low within the tiff image. The result is that ImaGene does not know

whether white or black pixels are the high values. If all signal values are negative, which can be deduced through quantification or use of the intensity tool, then select Invert Values and re quantify. The Find Negative Spots option is not intended for use in these situations.

- Enforce Grid Constraints – With this option selected, ImaGene will use Local and Grid Flexibility when performing spot finding. When not selected, ImaGene will perform spot finding with no constraints. Please see below for additional information regarding Local and Grid Flexibility.

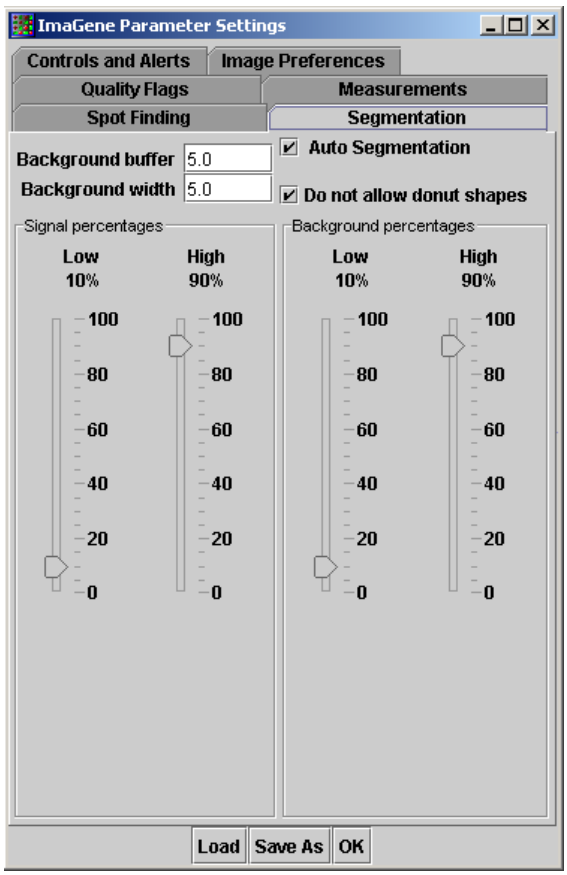
  Note: Under most circumstances, you will want to enforce grid constraints as this will limit the movement of circles during spot finding and help prevent erroneous spot finding due to dust and other contaminations.

- Local Flexibility – Local Flexibility defines the radius, measured in pixels, that ImaGene is allowed to search for spots. The origin for the search is the initial spot location as determined by grid placement. From here, ImaGene will search for a spot with *X* pixels distance where *X* is defined in the local flexibility parameter.
- Grid Flexibility – Grid flexibility is an indication of the extent to which ImaGene should deform the grid to match a given set of spots. The measurement is a qualitative notion based on a large part due to the unique properties of the image. Most users should set this to the middle values.

  Note: You can press the End key to move the slider all the way to the right or the Home key to move the slider all the way to the left.

Segmentation

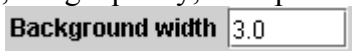
Segmentation is the differentiation of signal and background values within the array image and constitutes one of the most important aspects of array image analysis. Proper segmentation yields more robust data free from contamination and other adverse affects.



ImaGene supports two primary type of segmentation, manual and automatic. With Manual Segmentation, you must specify the appropriate values for ImaGene to use when analyzing the image. Often these proper values can be arrived at through experimentation or through experience. Alternatively, through a robust patent pending statistical approach, ImaGene can calculate the appropriate segmentation parameters for you. Under normal circumstances, BioDiscovery recommends use of the Automatic Segmentation due to its superior contamination removal. Regardless of the segmentation in use, both methods assist is in providing the highest quality data available.

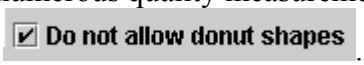
Listed under the Segmentation Tab are the following parameters:

- Background Buffer -

The distance, in pixels, between the signal and the background measurement regions. Within this region, all pixel values are ignored during quantification. Setting the proper buffer size helps to ensure accurate results. The desired size is dependent on several factors, including the spot size, density, image quality, and spot shape.
- Background Width - 

The measurement, in pixels, to determine how far background measurements will extend from the buffer region. In other words, the measurement will extend X pixels from the end of the buffer. Remember that the background should include enough pixels to provide a

sufficient sampling. ImaGene should not be used with no background values as these values are required in numerous quality measurements.

- Do not allow donut shapes - 

. When you select this option, ImaGene will make sure that a donut-shaped segmentation does not appear for any spot. A segmentation is considered to be donut-shaped if there are ignored pixels completely surrounded by signal pixels and their median intensity is lower then signal's. This option is available for auto segmentation only.

  Note: The background width will not extend beyond the snip, or rectangular boundary around each spot. Even though you can set the background to an extremely high value, the background measurements will stop at this boundary and not include signal values from surrounding spots.

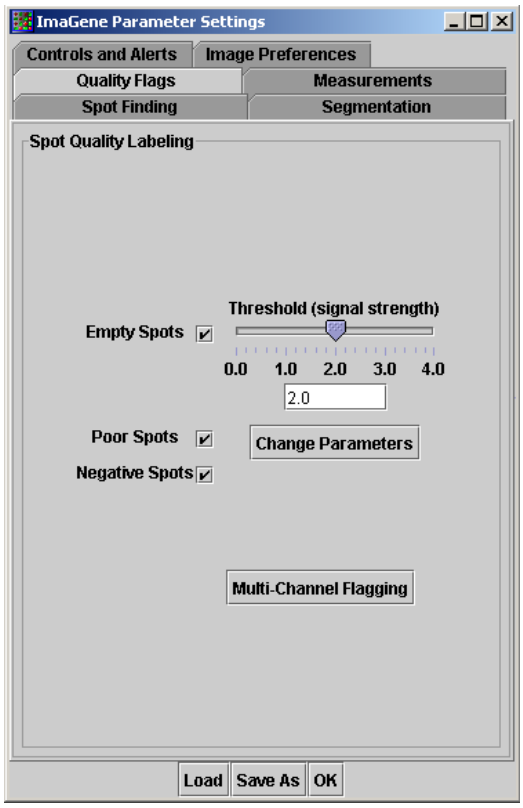
- Signal Percentages - These parameters are a percentage of all intensity levels within the signal region. The intensity ranges are raw values that do not include any statistical measurements (such as mean and median). The high percentage can be set as high as 100%, since the sample should contain the pixel with the highest intensity value. If you want to filter out possible noise sources, such as a speck of dust, set this percentage at a smaller value, like 95%. This way, the pixels associated with a dust particle, assuming they will be fluorescing at a high intensity level, will be filtered out.
- Background Percentages - These parameters are a percentage of all intensity levels within the background region. Set them the same way as described for the signal percentages. Press the Home key to set a slider to 0%. Press the End key to set a slider to 100%
- AutoSegmentation – Selecting this option will turn on BioDiscovery's patent pending automatic segmentation. Under most circumstances, this should be selected. Under AutoSegmentation, the only parameter, which ImaGene will use, is the Background Buffer. The typical value should be slightly less than ½ the radius. Under AutoSegmentation, the slider bars for the signal and background percentages become disabled.

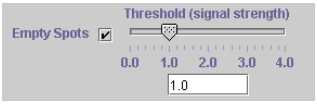
  Note: An important difference between automatic and manual segmentation is the fact that under manual, the parameters and their corresponding values are applied uniformly across all spots of the image. Under automatic segmentation, each spot is calculated independently usually generating more accurate segmentation.

Quality Flags

Once the segmentation is done and the spot expressions are extracted, the next question would be how much we can trust the expression data. In ImaGene the user can use various types of automated spot flagging schemas to help remove

suspicious spots from consideration. Alternatively, manual flags of different types can be applied for quality control.





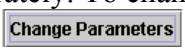
- Empty spots – this option allows you to automatically flag low-expressed spots or missing spots. This tool needs to have a sensitivity threshold set up. For each spot the following ratio is compared to the threshold.

$$R = \frac{\text{signal mean} - \text{background mean}}{\text{background standard deviation}}$$

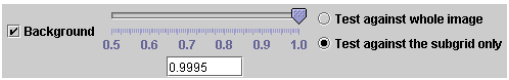
  If for some spot this ratio appears to be lower than the threshold value, the spot will be flagged with a green "X" mark on image. It will also have flag value 2 assigned in the corresponding column in results table. Threshold value can be chosen to be anywhere between 0 and 4. The user can either use a slider on Quality Flags panel of Settings dialog box or type the threshold value directly in the text field.
- Negative spots

– this tool will automatically flag any spot with signal mean lower than background mean. A green "-" mark will be issued to this spot on image and corresponding flag in the results table will have value 4.
- Poor spots

– ImaGene has a complex tool for detecting low-quality spots. It is designed to accommodate various possible interpretations of spot quality. This tool includes 7 different criteria for spot

fail/pass test that can be used in combination as well as separately. To change configuration of this tool, click "Change Parameters" button 

on Quality Flags panel of Settings dialog box. Once you click it, the new dialog box will pop up, and you will be able to select any combination of the following flagging tools.

1. *Flag for background being contaminated.* 

The top-most check box on quality parameters dialog corresponds to flagging method based on background abnormality detection. Background level of each individual spot is compared to local background level distribution over the image through a t-test. Resulting p-value is subtracted from 1 and is called "confidence in contamination presence". This confidence can be threshold at any level from 0.5 to 1 and the flag will be issued whenever particular contamination confidence goes over that threshold. Background mode is used as a background level estimate. Background level can be tested against information from all spots on the image or against information extracted only from those spots that fall within the same subgrid as the current spot. The latter approach can be useful if subgrids are large enough (>100 spots) and some kind of acceptable background level change is observed from one subgrid to another.
2. *Flag for signal being contaminated.*

Next check box corresponds to signal abnormality detection method. This approach is design to flag any spot with unusually high volatility in pixel-wise intensities within the signal area. For every individual spot signal variance is compared to the distribution of signal variance measurements across the image. However, before a t-test analogous to the one for background contamination is done, spots are grouped according to their signal mean measurements. This is done to compensate for a well-known observation that within-spot intensity volatility tends to be different for different expression levels. This way we make sure that each spot is tested only against spots close to it in their expression. The resulting confidence number is compared to the threshold analogously to previous procedure. Additionally, one can check "combine signal test and background tests" and two confidence numbers will be added together with equal weights 0.5 and compared to background contamination threshold.
3. *Flag for high ignored percentage.*

As it was mentioned before, some pixels may be assigned neither to signal nor to background in the presence of local contamination. Such contamination will be excluded from the measurements and called an "ignored" region. Ignored percentage flagging works as follows. It computes the area of ignored regions directly neighboring ("touching") the signal area. Let us denote it by I. Than the following ratio is computed.

$$R = \frac{I}{I+S} \times 100\%$$

S is the signal area in the above equation.

If for some particular spot R is higher than the pre-set threshold, this spot gets flagged for low quality.

4. *Flag for high open perimeter percentage.* ☑ Open perimeter percentage This tool is designed to catch a specific type of spot deformation. It computes the percentage of signal perimeter that touches the border of rectangular snip. Such a deformation can appear if the spot is significantly shifted from its expected position or if its shape has abnormal form. Whenever such percentage crosses the pre-set threshold, spot gets flagged as of low quality.
5. *Flag for abnormal shape regularity.* ☑ Shape regularity This algorithm computes a shape regularity – measure that characterizes closeness of spot's border to circular shape. First step of this algorithm is to inscribe a signal area of a spot into a circle. Than number of non-signal pixels that fall within this circle is computed and divided by circle's area. This ratio is subtracted from 1 as is called "shape regularity". Obviously such a variable will range from 0 (highly non-circular shape) to 1 (a perfect circle). Whenever this ratio falls below the pre-set threshold, spot gets flagged as of low quality.
6. *Flag for low Area-to-Perimeter ratio.* ☐ Area-to-Perimeter ratio This measure of spot's quality plays a role similar to the one of shape regularity just mentioned several lines above. Area of a spot is divided by a square of spot perimeter and multiplied by 4π . As a result, this measure ranges from 0 (highly non-circular shape) to 1 (a perfect circle). Whenever this ratio falls below the pre-set threshold, spot gets flagged as of low quality.
7. *Flag for significant offset from expected position.* ☑ Offset Expected position of a spot in current grid is computed by fitting least square lines to grid's columns and rows. Whenever the offset becomes larger than pre-set percentage of the distance between grid nodes, spot gets flagged for low quality.
   User can use any combination of the above quality measures with arbitrary thresholds. However, the quality parameters dialog has a Default button that allows to set a default configuration for the thresholds. Whenever at least one of selected criteria fails, spot is highlighted with "+" mark on image and receives flag value 3 in the results table.
   Note: You can use Multi-Channel Flagging button to change logic for combining automated flags between multiple channels. For all three types of automated flags (Empty, Poor and Negative) you can choose to assign a flag to a spot if it is flagged in at least one channel or in all channels.

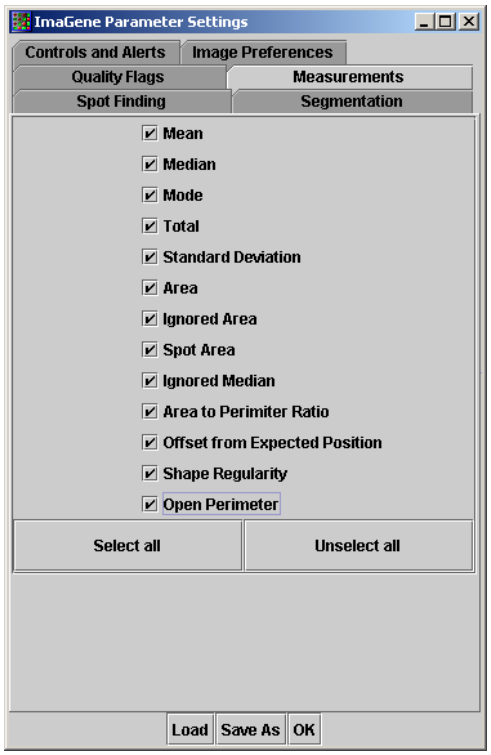
- Manual flagging – user can manually flag spots for the following reasons.

1. No particular reason – code 1, "X" mark
2. Empty spot – code 5, "X" mark
3. Poor spot – code 6, "+" mark
4. Negative spot – code 7, "-" mark

Manual flag can be placed using a Marking tool from the tool bar . Click on the Marking button, right-click on image to select a type of a flag. Place flags by clicking on corresponding spots. Manual flag marks will have red color.

Measurements

Various spot measurements can be computed by ImaGene and outputted to a tab-delimited text file. To select measurements that you are interested in you can use the Measurements panel of Settings dialog.



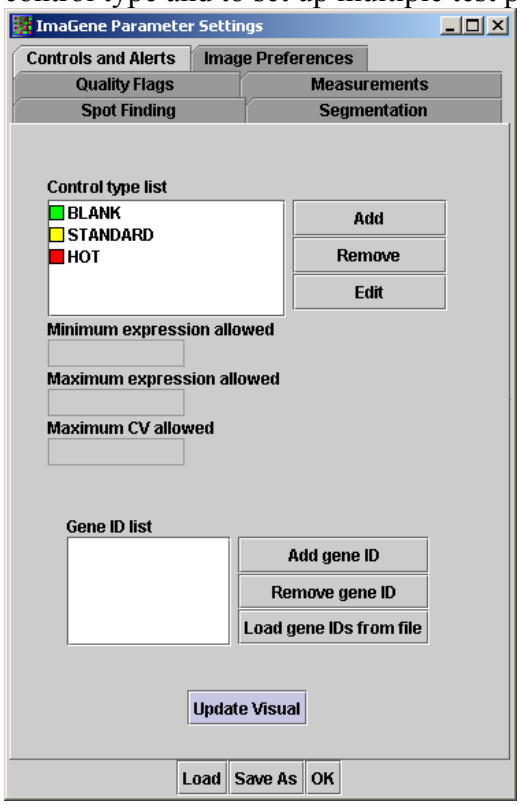
- Mean – signal and background mean intensity
- Median – signal and background median intensity
- Mode – signal and background mode intensity (mode corresponds to the pick of corresponding histogram)
- Total – signal and background total intensity (all pixel intensity summed up)
- Standard deviation – standard deviation of signal and background intensity distribution
- Area – number of pixels segmented as signal and background
- Ignored area – number of pixels in the areas segmented as ignored and directly touching signal area
- Spot area – ignored area plus signal area
- Ignored median – median of pixel intensity in ignored area
- Area to perimeter ratio – see “Quality Flags” section above
- Offset from expected position - – multiple offset measures get outputted, including X-, Y- and overall offset from expected position for the spot’s adjusted circle and spot’s center of mass.
- Shape regularity - see “Quality Flags” section above

- Open perimeter – see “Quality Flags” section above, measure is outputted as a decimal fraction corresponding to Open perimeter percentage (0%=0.0, 100%=1.0).

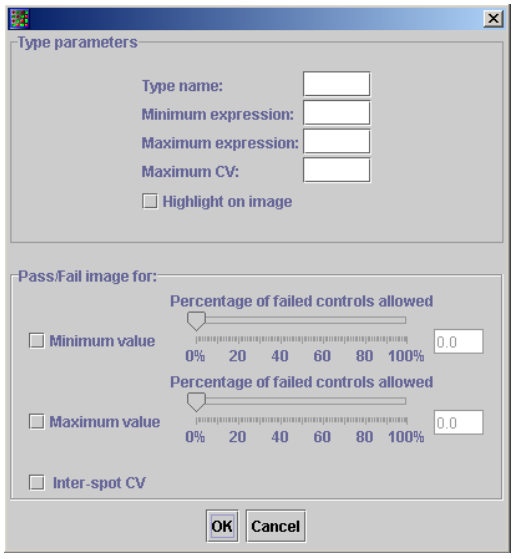
  User can check and uncheck these measurements at any time; results table will be updated immediately.

Controls and Alerts

ImaGene incorporates a powerful tool for testing expressions for control spots located on image. This tools allows to group several gene IDs into a unique control type and to set up multiple test parameters for the type.



Using Add button on the panel user can create a new control type by specifying several parameters.



- Type name – provide arbitrary name for the type
- Minimum expression – provide minimum value for signal mean for the type
- Maximum expression – provide maximum value for signal mean for the type
- Maximum CV – provide maximum coefficient of variance between signal means for the spots within this type (should be >0).
- Highlight on image – specify if you want all spots of this type to be highlighted on image. If you select this check box, you will be able to select a color for presentation of this type. Spots of this type will be marked with squares; the problematic spots corresponding to violation of above thresholds will be highlighted with diamond shapes inside the squares.
- Pass/fail image for minimum value – whenever the number of spots that belong to this type and violate the minimum expression criterion becomes higher than specified percentage, the warning box will pop up after the quantification procedure is completed or Update Visual button is pressed.
- Pass/fail image for maximum value – whenever the number of spots that belong to this type and violate the maximum expression criterion becomes higher than specified percentage, the warning box will pop up after the quantification procedure is completed or Update Visual button is pressed.
- Pass/fail image for inter-spot CV – whenever the inter-spot CV of signal means within this type violates the maximum CV criterion, the warning box will pop up after the quantification procedure is completed or Update Visual button is pressed.

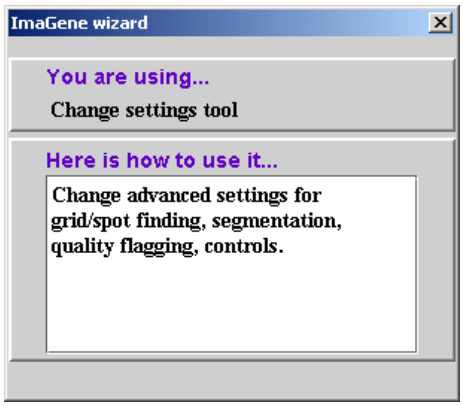
Whenever the new type is added, its name shows up in the type list. Next step would be to add gene IDs that belong to this type. Select a type from the list, click on “Add gene ID” button. A new dialog box will pop up. If gene ID file is loaded, you can select any gene IDs from the list on the right-hand side of the dialog and click Add. You alternatively may just type an ID in the text field and click Add. In this field you can also type a key for assigning gene IDs. The key is created using a “*” symbol. For example by adding a key “BLANK*” you assign all gene IDs that start with “BLANK” to the current control type. Whenever you are done adding gene IDs, click Close.

Alternatively, you can use

button to load a list of gene IDs from a text file. Such file can contain a column of multiple gene IDs, one gene ID per line. Once loaded, such gene IDs will be assigned to the current control type. This feature is useful if you want to bring a list of "selected" genes from some "alien" program (GeneSight for instance) and analyze corresponding spots on microarray images.
To see all spots that belong to the specified types highlighted on image, click Update Visual. This action will also show all control violations. If current image failed the controls test, corresponding warning box will pop up. Control type for every spot will also be shown in the results table in one of the columns.

1.5 ImaGene Wizard

ImaGene wizard is designed to help user understand ImaGene tools better and to assist in going through necessary steps. Wizard can be switched on and off at any moment through Help menu. Even when Wizard window is switched on, it does not limit user's access to any of ImaGene tools.


ImaGene wizard
You are using...
Change settings tool
Here is how to use it...
Change advanced settings for grid/spot finding, segmentation, quality flagging, controls.

To launch Wizard, select "Wizard On" option from Help menu. Wizard window will be shown in the bottom right part of your screen.

ImaGene Wizard works in two modes. First mode switches on whenever Wizard detects that user performed some action that should be logically followed by some other action. Then Wizard will prompt what action was performed and what should be done next. To follow this advice user can either use a tool Wizard refers to or just click on "Follow Advice" button in the bottom part of Wizard window. The latter will result in the recommended action being performed automatically. If recommended action is not necessary, user will have an option to go to next advice. Sequence of recommended actions will start immediately after the ImaGene launches with the advice to load the image. Thus, virtually any user can just follow the Wizard prompts to learn how to use ImaGene.

If user tries to apply some tool that does not fall into a pre-defined sequence of actions, Wizard will switch to its second mode. In this mode Wizard will just prompt the user what tool is being used and how to use it.

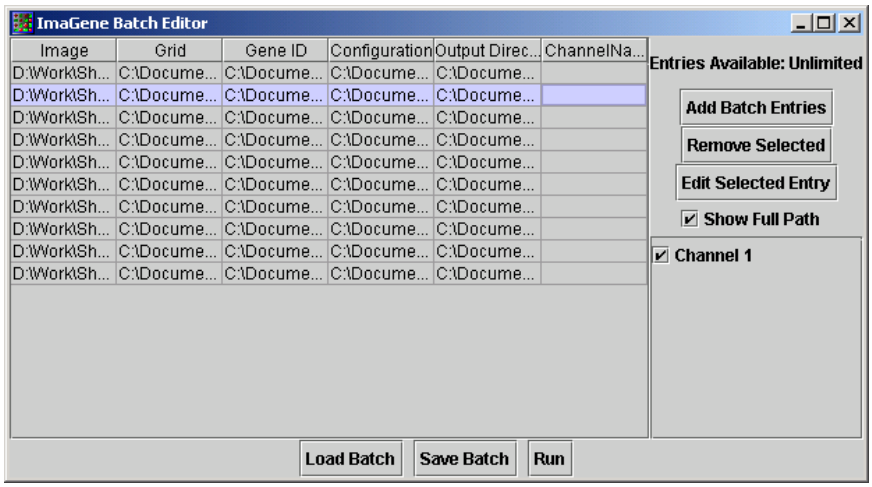
Using ImaGene Wizard user has an opportunity to learn how to use the software in an interactive fashion.

1.6 Batch Editor and Batch Processing

The ability to batch process images is an optional component that can be added to the base ImaGene. A batch is simply a series of similar images to be processed with the same settings to be processed autonomously. Through the use of this module, ImaGene can be used in high throughput environments where large numbers of arrays are processed everyday. The same data is exported from ImaGene whether run in batch mode or not. During batch mode ImaGene display a simple, intuitive progress window indicating information about the current image and state of the batch.

1.6.1 The Main Batch Editor Window

The ImaGene Batch Editor Window allows batches to be creates saved and loaded. Batched can be created and saved for later use or as a template to be modified at a later date. Even details such as which channels of mutichannel/image composite to process can be selected.



Batch Table
Once a batch has been created, the information about the batch is displayed. The following information is contained within the table:

- Image – The name of the image
- Grid – The name of the grid

- Gene ID – The name of the Gene ID file
- Configuration – The name of the configuration file
- Output Directory – The name of the output directory
- Channel Name – The Channel to be processed when using composite images.

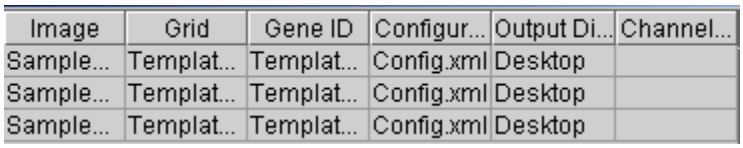


Image	Grid	Gene ID	Configur...	Output Di...	Channel...
Sample...	Templat...	Templat...	Config.xml	Desktop	
Sample...	Templat...	Templat...	Config.xml	Desktop	
Sample...	Templat...	Templat...	Config.xml	Desktop	

Buttons

From within the Batch Editor Window, buttons exist to do the following:

- Add Batch Entries – Adding batch entries allows several images to be associated with a settings and grid file for processing. For example, if I want to add 10 images to be processed, then I would only need to click this once, specify the 10 images, some other parameters and the 10 images would be added to the batch. If I then had 13 more images of a different type, I would repeat this process of adding batch entries until all images were added. As I add batch entries, the information is displayed within the Batch Table to the left of the button.
- Remove Selected – This will remove the selected entry from the batch. Multiple entries may be highlighted and removed through use of the <shift.> and <ctrl> keys.

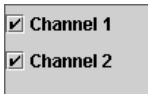
- Edit Selected Entry – This opens a window that allows the batch parameters to be changed. For example, if the location of the images changes, this can be used to update this field. If a batch file template is made, this tool would be used to modify any default values, if need be.
- Load Batch – Load Batch loads from the file systems a batch file that has been previously saved. ImaGene batch files are simple XML files with a .bch file extension. Please see Section 5.1 regarding ImaGene file specification for additional information on ImaGene batch files.
- Save Batch – The batch, including information about all the entries will be saved to a file on the hard disk. This file can later be reloaded via the Load Batch Button.
- Show Full Path – By default, ImaGene will only display the name of the files within the Batch Table. Select this option to display the entire path within the table. For example, select this checkbox to display the Grid file as C:\ImaGene\Batch\ImaGeneGridFile.grd.

- Entries Available – This field indicates the total number of images that are available to be processed. For most users, the field will indicate an unlimited number of images to be processed.

Note: When saving the batch, only basic information about the location of configuration files and image files is included. The actual files remain unchanged in their original location. Do not confuse saving the batch file with saving the configuration files used to run the batch.

- Run – The batch and all its entries will be started. Soon after clicking Run, the Batch Editor Window will disappear and the Batch Progress Indicator becomes visible.

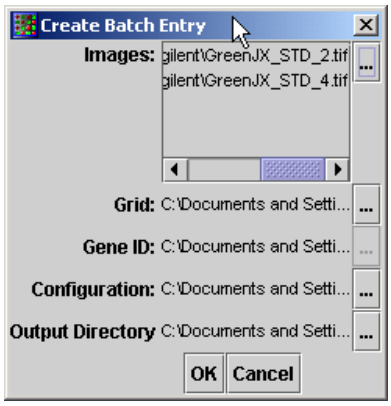
Channel Selector
Located along the bottom right of the ImaGene Batch Editor Window is the Channel Selector. The channel selector is designed for use with multichannel images, also known as composite images. Essentially, a composite image is a single file, which contains several images within it. This makes it possible to load a single file within ImaGene, yet have 2, 4 or more images then visible. Several scanner manufacturers on the market today produce such file formats. While most of the scanner software can also export the images in a single, one image per file format, ImaGene is flexible enough to support both formats.



There may be times when only a single channel of a multichannel scan is of interest to you. If this is the case then only processing the desired channel may save time.

1.6.2 Create Batch Entry Window

The Create Batch Entry Window allows entries to be added to a batch. The window appears as a result of clicking either the Add Batch Entries Button or the Edit Selected Entry Button. The window requires four parameters to be entered in order to batch a set of Images. All images, which belong to a common set, can be created here and added to the batch as a group. For example, if I have 5 images generated with Molecular Dynamics hardware, then I may wish to add all five images here since they contain the same settings and grid within ImaGene. This allows me to specify a single settings file and template file for several similar images.



The required parameters include:

- Images – The images to be included with the batch and to be associated with the setting mentioned next. Typically, while the hybridization information about the images may be unique, the geometry of the arrays is the same. If this is the case, then multiple images can be loaded here.
- Grid – The grid file that matched the images to be batched. A template file may also be used as this includes a grid. If a template file is used, then a gene ID file does not need to be specified.

  Note: A template file contains the grid as well as the corresponding gene ID info. I grid file does not contain gene IDs.

- Gene ID – The Gene ID file allows you to track information about the genetic material spotted at each location within the array. This information will be saved along with the quantified values in the text output file and visualization tools. Please see section 5.1 for the proper file specification for a ImaGene gene ID file.
- Configuration – The configuration file contains information about the parameters and their corresponding values used during analysis within ImaGene. Common examples of stored parameters include local flexibility for spot finding and the desired quality measures for the text output file. The file itself is a simple XML file that can be saved and loaded to the ImaGene Parameter Settings Window.
- Output Directory – ImaGene requires the location where to deposit the subsequent text files after analysis. Click, browse to and select the output directory to use. The location can be either be local or on a network drive.
- OK – Once all the required parameters have been specified the OK Button becomes enables and the entries can be added to the batch.
- Cancel – The window is closed and the entries are not added to the batch.

1.6.3 Creating and Running a Batch

Setting up batch processing within ImaGene takes only a matter or minutes and requires only a few pieces of information. The description below covers the basics of creating and running a batch as well as common tips and tricks for success.

1. Setup the initial parameters for use within ImaGene. This needs to be done the first time prior to running a batch. To this perform the following:
a. Load a sample image
b. Load a gene ID
c. Create and Place a grid on the image
d. Save the result as a template to a location of your choosing
e. Set the parameters on the ImaGene Parameter Settings Window
f. Save this configuration to disk. You will probably want to save this to the same location as the template previously created.
2. From the ImaGene Menu Bar select File then Batch Editor.
3. Click the Add Batch Entries Button
4. Specify the images to be batched
5. For Gird, specify the template previously created.
6. Likewise, for configuration, specify the configuration previously saved.
7. Specify the output directory. This is the directory, which will store both the data and the snapshot file (or sst file).
8. Click the Run Button

1.6.4 Saving a Batch

Once entries have been added to the batch, the batch can be saved and recalled later. To save the batch to a file, perform the following:

1. Click the Save Batch Button
2. In the Save As Dialog that appears, navigate to the location where you wish to save the file
3. Specify the name of the file. A .bch file extension will automatically be added to the name.

1.6.5 Loading a Batch

If a batch file has been saved to the file system, it can be easily recalled and used again. The following steps outline loading a batch file:

1. Click the Load Batch Button
2. Browse to and select the desired batch file
3. Select Open to load the file
4. The batch entries are now visible within the Batch Editor.

1.6.6 Editing a Batch

Once entries have been added to a batch they can be easily modified, eliminating the need to start anew creating the batch file. To modify an existing entry within the Batch Editor follow these steps:

1. Select, or highlight, the desired entry by left clicking on its row within the Batch Table with the left mouse button. Only one row at a time may be modified.
2. Click Edit Selected Entry Button to open the Create Batch Entry Window for editing.
3. Modify the desired parameters
4. Click OK to commit the changes or cancel to return to the Batch Editor without any changes.

1.7 Reviewing Results

One of the most powerful features of ImaGene is the ability to review results of previously processed data. ImaGene is designed to load and display data exactly as it was processed days or even months earlier The capability to review the results provides the following benefits:

- Compare old data with recently processed data
- Review the results of batch processing
- Establish post image analysis quality controls and screening
- Reload and take screen captures for publication

1.7.1 The Snapshot File (sst file)

The snapshot file is a result of quantification within ImaGene. Upon saving the quantified data to the file system, ImaGene also saved another file, which contains a snapshot of that data as it existed after quantification. It is this snapshot file, ending with an .sst file extension, which ImaGene loads and allows for results to be reviewed.

The snapshot file itself is simply a proprietary binary file format which is not useful outside of ImaGene. Due to the large amount of information contained within the file, its size can grow quite large, often surpassing the image size itself. The snapshot file contains the following components within it:

- Grid
- Gene ID
- Segmentation
- Quantified Data
- Quality Measures
- Flag Values

While the sst file contains almost all the required information to reload and review data, it does not contain the actual images. To review results, ImaGene must have access to the original images. By default, ImaGene will attempt to load the images from the location specified within the sst file. However, if the images have been moved and ImaGene is not able to load them, ImaGene will ask you to browse to and select the appropriate images.

1.7.2 Loading Results to Review

Loading data for subsequent review can be accomplished by performing the following steps:

1. From the ImaGene menu bar, select File followed by Review Results. Likewise, you may click on the Review Results icon located along the toolbar.
2. With the Open dialog, browse to and select the snapshot file you wish to open.
3. Depending on the file, ImaGene may prompt you to specify the location of the original images as well.
4. The information and data should now be visible within the main ImaGene user interface. If you wish to reprocess the images, click the Cancel button along the bottom of the Results tab and use Quantify button of the main panel.

1.8 ImaGene Tools

ImaGene includes many handy tools designed to help facilitate array analysis. These tolls are designed to allow manipulation, quantification and analysis of array images.

1.8.1 Auto Alignment Tool

The Auto Alignment tool automatically overlays several images on top of one another. If two or more images are loaded, activating this tool will attempt to overlay the spots from each image. While this tool is designed largely for transposing of images, or left, right, and up, down, movement, ImaGene cam handle up to 10 degree's of rotation as well.

The Auto Alignment tool is intended to provide quick and highly accurate means to solve a common problem of array image analysis, arrays which have been scanned slightly out of alignment. If the images have a high degree of rotation or another extreme problem, then the manual manipulation tools of transposing and rotation may be used.

Note: The Auto Alignment tool is intended to account for shifts of the array in scanning and not for warping of the medium being printed upon.

1.8.2 Auto Grid Placement Tool

ImaGene's Auto Grid Placement Tool automatically repositions a grid structure to the proper location within the image. A common application of this tool is with the use of grid and template files. Of the geometry of an array will stay the same through and between experiments. If this is the case, then the use of a common template for all experiments would be beneficial. However, due to differences in arraying and scanning, the location of the array within the image may vary across experiments and time. The solution is to use the Auto Grid placement tool once the image and the grid have been loaded. The tool will automatically move the grid to the proper location.

Because the Auto Grid Placement Tool is based on the same grid placement algorithm that Batch Processing uses, the tool is also beneficial to test grid placement on a set of images prior to setting up a batch. For example, instead of wasting time trying to process a poor quality image through the batch processing module, first try the Auto Grids placement on the image. If the Auto Grid Placement cannot locate the proper array geometry then the mage will probably need to be processed manually.

1.8.3 Save Display Image Tool

ImaGene'S save Display Image Tool provides the capability to save a screen capture of the overlaid, i.e. composite, images. The saved image includes only the overlay as it appears within the main image panel. ImaGene saves the image as a high quality 24 bit tiff format. The image can later be recalled for reference of use in publications.

1.8.3.1 Saving the Display Image

Perform the following step to save the current composite overlay to a tiff image file:

1. From the menu bar select File then Save Display Image. Alternatively, click the Save Display Image icon from the toolbar.
2. From the Save As Dialog that appears, browse to the location you wish to save the image.
3. Specify a file name. ImaGene will automatically add the .tif file extension at the end of the name.
4. The image is now saved and is available to be viewed in virtually any graphics program such as Microsoft Imaging or Adobe Photoshop.

1.8.4 Zoom Tool

The Zoom Tool , as its name implies, allows you to zoom a specific region of the image. The several methods which ImaGene currently supports to zoom include:

- After selecting the Zoom Tool, left click with the mouse and drag a rectangle around the section of interest from within the main image panel.
- Similarly to above, After selecting the Zoom Tool, left click with the mouse and drag a rectangle around the section of interest from within the Image Map View.
- Hold the <alt> key on the key board, left click with the mouse and drag a rectangle around the section of interest from within the either of the areas mentioned previously.
- Drag the Zoom slider bar located directly below the Main Image Panel.

Note: To return to the original image size, double right click with the mouse.

1.8.5 Scroll Tool

The Scroll Tool provides a convenient way to move about the image without requiring zooming out from the current level. To pan the image, simply click and drag the image around as you would any other object. The image will automatically scroll left and right as needed.

If at any time you wish to know where within the image the current view is situated, simply look for the location of the yellow box within the Map View. The yellow box indicates the region currently being viewed within the Main Image Panel.

1.8.6 Ruler Tool

The Ruler Tool allows objects to be measured within the Main Image Panel. The most common use of this tool is to determine the minimum and the maximum diameter for spot sizes and distance between the spots. The tool can also be used to determine the general resolution of the image and how many pixels are included within each spot.

The Ruler Tool displays the results within the Status Bar located under the Main image Panel. The information provided includes:

- Dx: the distance moved on the x-axis as measured in pixels
- Dy: the distance moved on the y-axis as measured in pixels
- distance: the absolute distance moved as measured in pixels

1.8.7 Intensity Tool

The Intensity Tool displays the raw pixel intensities within the Status Bar located under the Main Image Panel. After selecting the tool, simply position the cursor over the pixel of interest and the pixel intensity for each image is displayed.

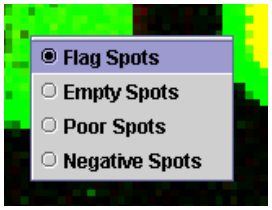
Note: The values provided here are raw intensities meaning they are not mean or median values but rather a single pixel value.

1.8.8 Flagging Tool

The Flagging Tool provides a simple means of manually flagging spots within ImaGene. While spots may be flagged or marked for a variety of reasons, the most common reason is due to defect within the spot or array.

Once a spot has been flagged, a numerical value indicating the type of flag is then outputted with the quantified data. The flag value can then be used in later data analysis software, such as BioDiscovery's GeneSight, or can be used in post analysis quality control measures. In addition to the numerical value, a shape is also added to make the flag visually unique. Each shape corresponds to a different flag and is explained below.

ImaGene supports a variety of automatic and manual flagging types. To change the type of flag, right click with the mouse and select from the context sensitive menu the desired manual flag. The following is a list of possible flags and the corresponding values:



Flagging codes:

Manual flags (color: red)
1 - Flag spots
5 - Empty spots (cross)
6 - Poor spots (plus)
7 - Negative spots (dash)

Automatic flags (color: green)
2 – Empty spots (cross)
3 – Poor spots (plus)
4 – Negative spots (dash)

Note: Manual flags override auto flags.

1.8.9 Undo and Redo Tools

ImaGene supports an unlimited number of undo and redo operations. Anytime you wish to go back and not do an action, this tool will provide means to do so.

Note: The action to be undone or redone is displayed next to the text within the menu bar. For example, if I move the grid then wish to undo this action, from within the Tools Menu, the text will read "Undo Move Grid". This is a convenient means to see what action will be reversed.

1.8.10 Grid Tools

Adjust Metagrid
This tool allows manipulation of entire metagrids. The metagrid can be moved, stretched or resized.

Adjust Subgrid
This tool allows manipulation of entire subgrids. The subgrids can be moved, stretched or resized.

Adjust Spot
This tool allows for individual spots to be moved and resized.

Lasso Adjust
This tool allows you to select irregularly shaped regions of circles for adjustment. Instead of shifting entire subgrids, you can now specify unusually shaped regions of circles for fine-tuning. For example, you would want to use this tool with membrane arrays because edges could become warped quite often.

Rectangle Adjust
This tool, which is similar to the lasso adjust tool, allows you to make a sub-selection of circles to adjust from within the array. However this tool employs a rectangular shape to highlight given circles. A possible use of this tool would be with microarrays produced by using a bent pin mounted on the pin head. In such a case a definite rectangular anomaly exists within the array making it ideally suited for the Rectangle Adjust tool.

Duplicate MetaGrid
Duplicating an existing metagrid can save time and produce better results rather than manually recreating the metagrid several times. To use it click on the button and select a metagrid to duplicate by left-clicking on it. After that you can multiply this metagrid in any place of image by left-clicking there. To stop copying make a right button click.

Show/Hide Grid
ImaGene allows the grid to be hidden enabling a better view of the bare spots. Hiding the grid hides any lines, circles and flagging that may exist within the Main Image Panel.

Auto Adjust Spots
Select this option to have ImaGene perform automatic spot localization. ImaGene searches the area around grid placement for a spot within the predefined minimum and maximum diameters. Adjustment accuracy also depends on grid constraints and the quality of your images. For example, if you have spots with irregular signal values and you set grid flexibility with a high number of pixels, ImaGene will tend to find pixels with high expression values. For full automation use this tool in conjunction with auto grid placement button.

Note: If you use this button several times, spot searching will start from the position where previous attempt left it. Be careful: multiple auto adjustment can dramatically deform grids on low-quality images.

Wrangle
The wrangle feature of ImaGene applies new, stricter constraints to the results of spot localization without requiring further spot finding. Essentially, this allows users to reduce the spot search radius without redoing the spot finding. The benefit of this feature is to assist processing for those with either slower computer hardware or for those with numerous spots.

A sample application would be to perform spot finding for grid geometry on an array image with a local flexibility set to a large number of pixels. After spot finding, if the resulting circle placement has high variability, the Local Flexibly can be reduced. After reducing Local Flexibility, click the Wrangle Button to apply the new setting without waiting for spot finding to be performed again.

Part 2: ImaGene Tutorials

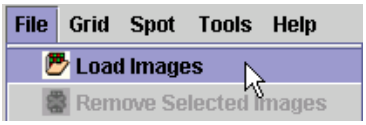
2.1 Basic Analysis

This tutorial demonstrates performing basic quantification through ImaGene. The tutorial covers the essential steps from loading the initial array images until a report is generated via GeneSight-Lite. The following lessons will be presented within this tutorial.

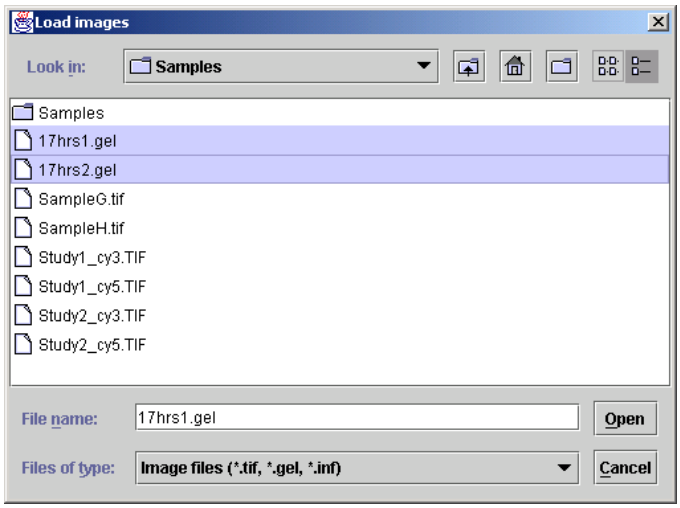
- **Image Loading and Manipulation** - Describes how to load an image into ImaGene and use the controls on the Image tab to enhance the on-screen display of the image.
- **Grid Placement and Manipulation** – Describes how to create a grid and adjust it to fit the grid properly.
- **Quantifying and Saving the Data** – Describes the quantification and subsequent output of data.
- **Generating a Report Via GeneSight-Lite** – Describes report generation using ImaGene built in data analysis module GeneSight-Lite

Image Loading and Manipulation

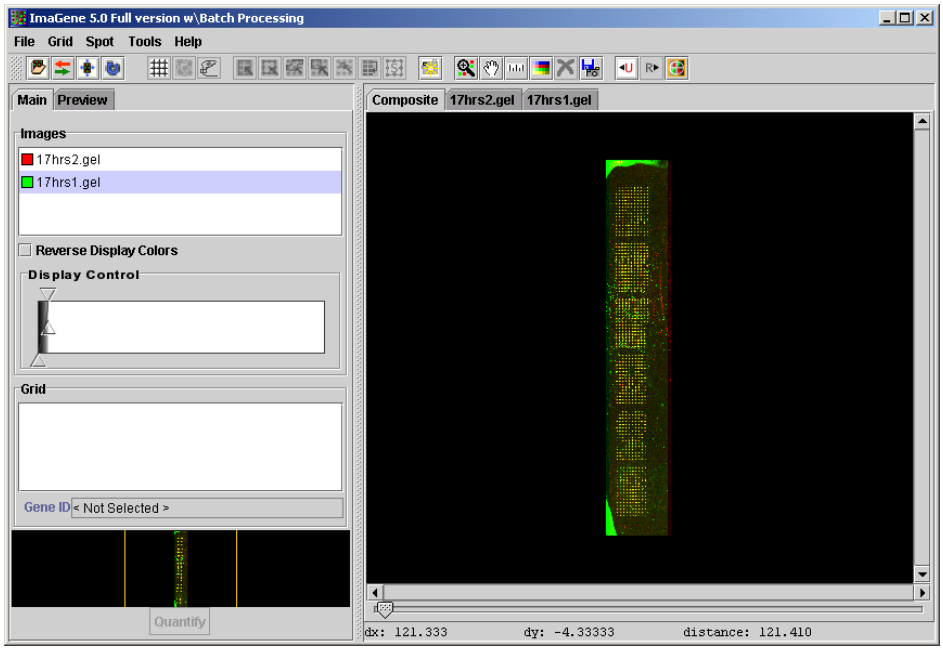
1. Launch ImaGene from the desktop icon or from the ImaGene folder within the Windows Start Menu.
2. From the menu bar, select File followed by Load Images.



3. Browse to the Samples folder within the ImaGene folder. The location is typically, C:\ImaGene\Samples.
4. Select 17hrs1.tif and 17hts2.tif. Multiple selections can be performed by holding the <ctl> key on the keyboard and left clicking.

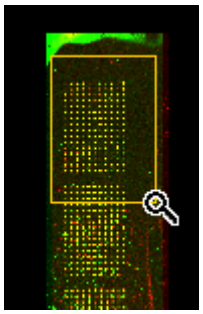


5. Click the Open Button to complete the image loading. A progress bar displays time remaining.
6. Once the images are loaded and are visible within the Main image display located along the right of the primary ImaGene window, the images need to be overlaid. Not all images will require this action, but due to variances in scanning, some will. To overlay the Images Automatically, click the Align Images button from the toolbar. In additional to auto alignment, ImaGene offers manual tools for rotation and translation of images.

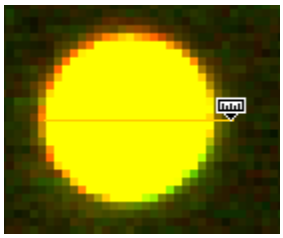


Grid Placement and Manipulation

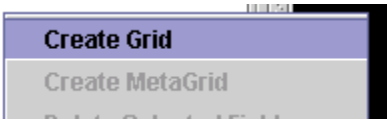
7. From the menu bar, select Tools followed by Zoom.
8. Drag a rectangle around the top subgrid of the array to zoom into this region of the image.



9. Select Tools then Ruler from the menu bar to measure the approximate sizes of the spots within this array. This information will be used slightly later.
10. With the ruler tool, click and drag from one side of a spot to the other side of the spot. The distance is displayed in a status bar located beneath the main image panel. This distance is in pixels.

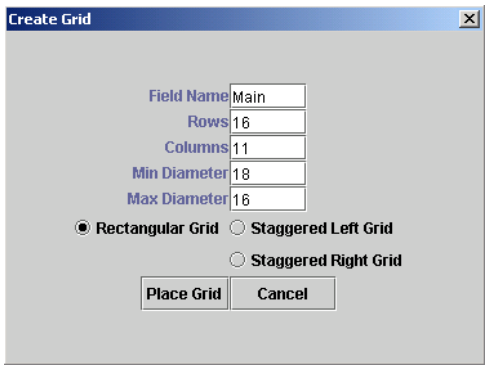


11. Next count the number of rows and columns of spots within this subgrid.
12. Right click on the Grid Panel and select Create Grid from the menu that appears.

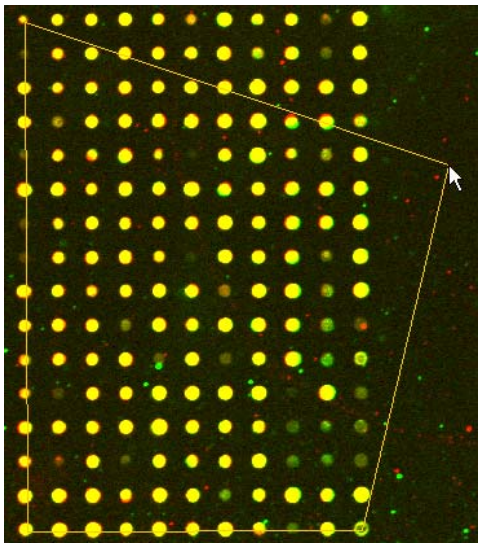


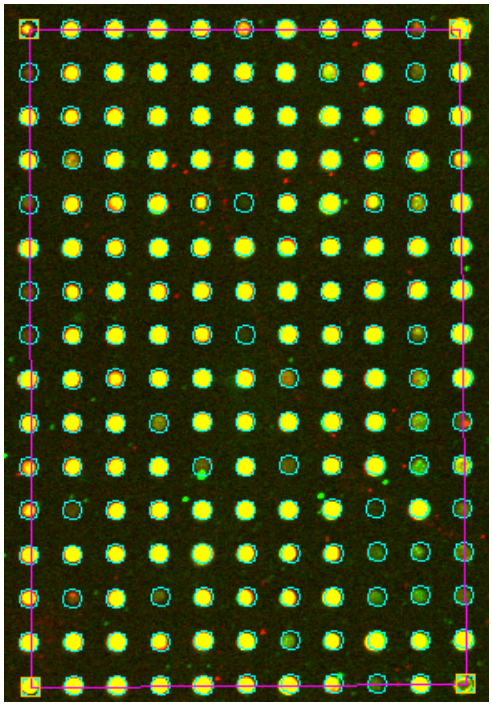
13. Enter the following information into the Create Grid Window that appears.

a. Field Name - : "Main"
b. Rows – "16"
c. Columns – "11"
d. Min Diameter – "18"
e. Max Diameter – "26"
f. Rectangular Grid is selected

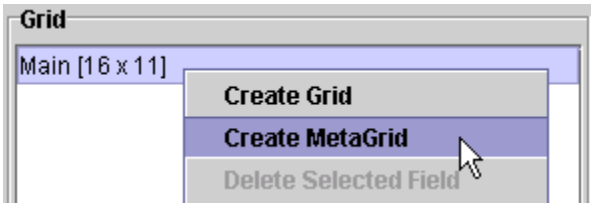


14. Click the Place Grid Button
15. Use the mouse cursor and click on the four corner spots of the subgrid. Upon placement of the fourth spot, the grid becomes visible on the subgrid.

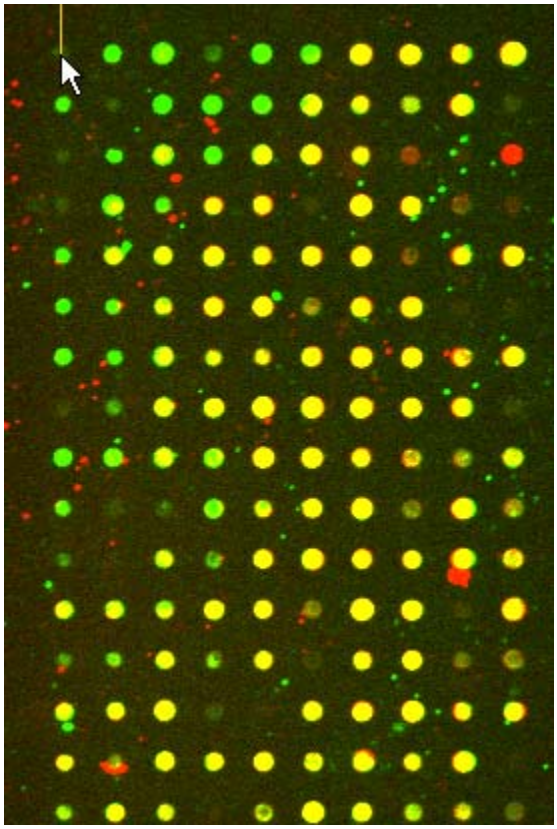




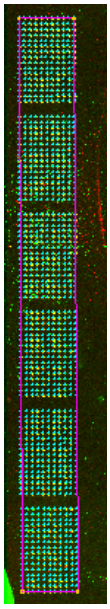
16. Click on the name Main in the Grid Panel.



17. With “Main” selected, right click and select Create Metagrid.
18. In the window that appears, enter the following information
a. Metarows – “6”
b. Metacolumn – “1”
19. Click Place MetaGrid
20. In the top subgrid where we just placed the grid, click on the top, left most spot.
21. Using the scroll bar located along the right of the main image panel, scroll downward until the last subgrid is reached



22. Click the top, left most spot within this subgrid as well. ImaGene automatically places the remaining subgrids in the proper locations.

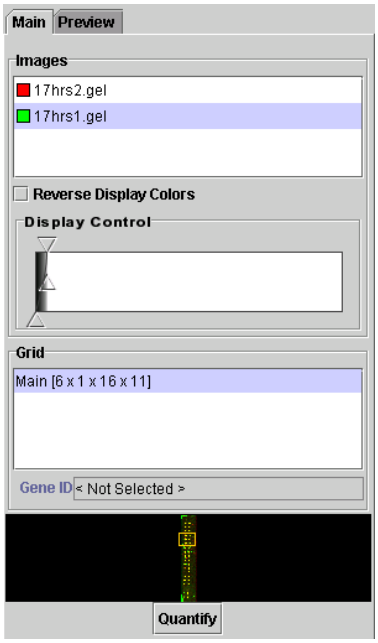


23. Using the scroll bar along the right of the main image panel, scroll up so that the second to last subgrid is visible.

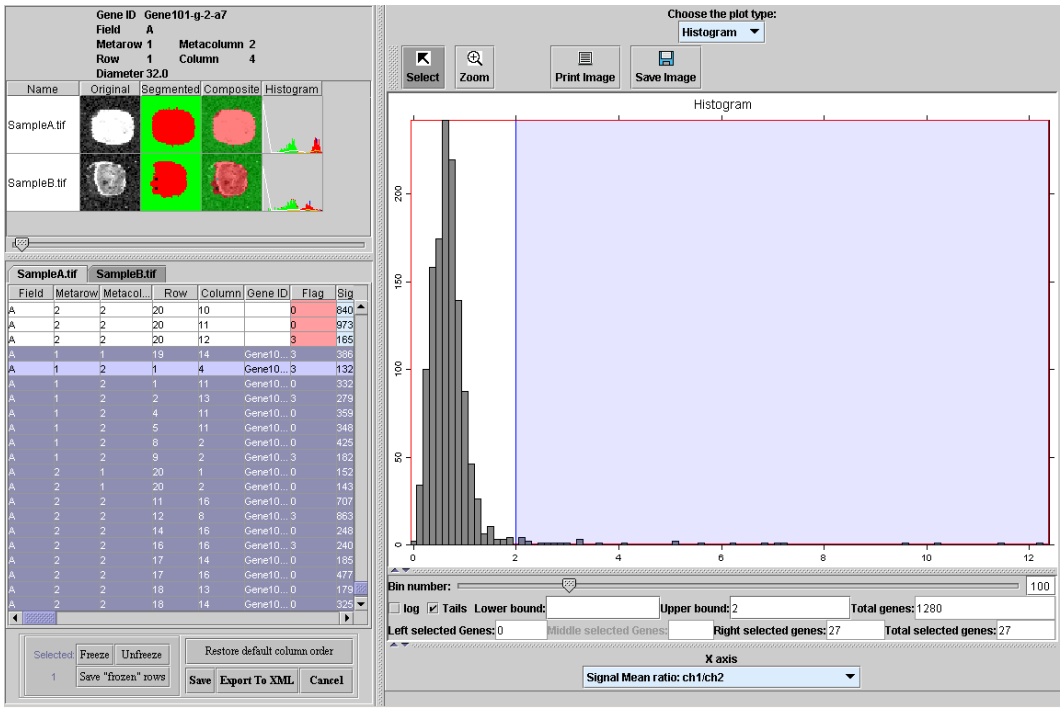
24. Click the Adjust Subgrid button on the toolbar.
25. Click on and drag this subgrid slightly to the left to provide a better fit.
26. Or alternatively click the Automatically Place Grid button and the subgrids will be adjusted automatically.
27. Click the Adjust MetaGrid Button from the toolbar and click on any subgrid.
28. Click the Auto Adjust Spots Button from the Toolbar to perform automatic spot finding.

Quantifying, Pre-Analyzing and Saving the Data

29. Click the Quantify Button located along the Bottom of the Main Tab.



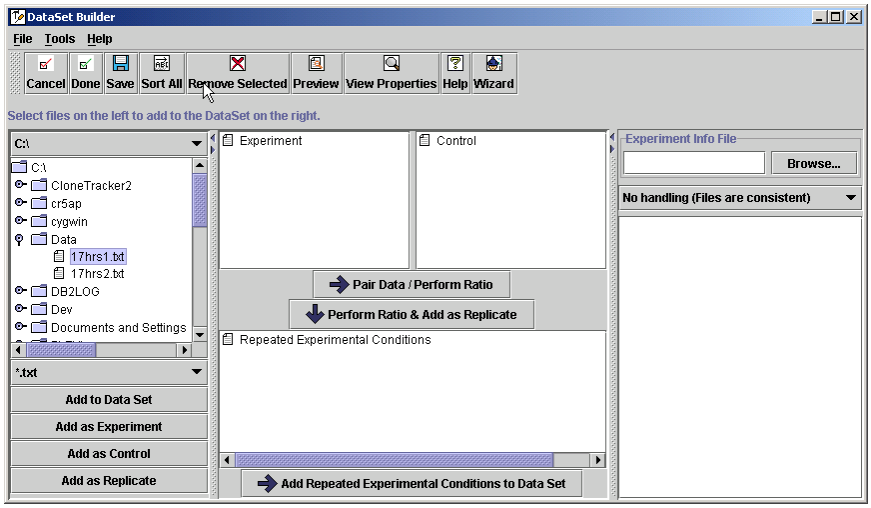
30. After a few moments the quantified data is visible under the Results Tab.
31. Select Plots tab on the Image Display panel.
32. Select Histogram on the Plots panel.
33. Select “Signal Mean Ratio ch1/ch2” from the list of measurements.
34. Make sure that “log” option is not selected for the Histogram.
35. Select 2-fold regulated genes by specifying 2 in the “Upper bound” text box.
36. “Freeze” the selection using corresponding button in the bottom of the table.
37. Sort the data by column “Selected” clicking on its header. All selected genes will be in the bottom of the table.
38. Go through the spots one by one analyzing spot images.


Gene ID Gene101-g-2-a7
Field A
Metarow 1 Metacolumn 2
Row 1 Column 4
Diameter 32.0
Name Original Segmented Composite Histogram
SampleA.tif
SampleB.tif
SampleA.tif SampleB.tif
Field Metarow Metacol... Row Column Gene ID Flag Sig
Selected: Freeze Unfreeze
Save "frozen" rows
Restore default column order
Save Export To XML Cancel
Choose the plot type:
Histogram
Select Zoom Print Image Save Image
Histogram
Bin number: 100
log Tails Lower bound: Upper bound: 2 Total genes: 1280
Left selected Genes: 0 Middle selected Genes: Right selected genes: 27 Total selected genes: 27
X axis
Signal Mean ratio: ch1/ch2

39. Click the Save Button to save the Quantified data and snapshot file to an output directory of your choice.

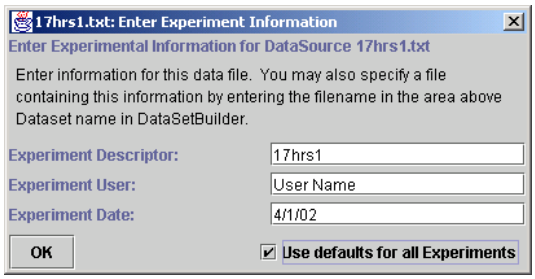
Generating a Report Via GeneSight-Lite

40. If further analysis is needed, click the GeneSight-Lite icon from the toolbar. After a brief moment GeneSight-Lite and the Dataset Builder will appear. (For complete information on GeneSight-Lite, please see the GeneSight User's Manual.)
41. With the file system window along the left of the Dataset Builder, browse to the data file previously generated within ImaGene.

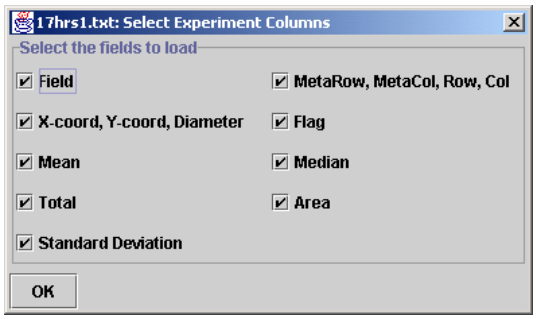


42. Select and drag 17hrs1.txt to the Experiment Panel.
43. Select and Drag 17hrs2.txt to the Control Panel.
44. Click the Pair Data / Perform Ratio Button.
45. Click Done from the Dataset Builder Toolbar.

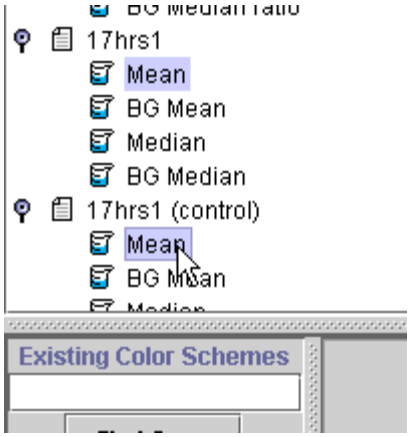
46. Select User Defaults for all Experiments and click the OK Button.


17hrs1.txt: Enter Experiment Information
Enter Experimental Information for DataSource 17hrs1.txt
Enter information for this data file. You may also specify a file containing this information by entering the filename in the area above Dataset name in DataSetBuilder.
Experiment Descriptor:
17hrs1
Experiment User:
User Name
Experiment Date:
4/1/02
OK
Use defaults for all Experiments

47. Click OK on the next window to use all experimental conditions available within the data files.

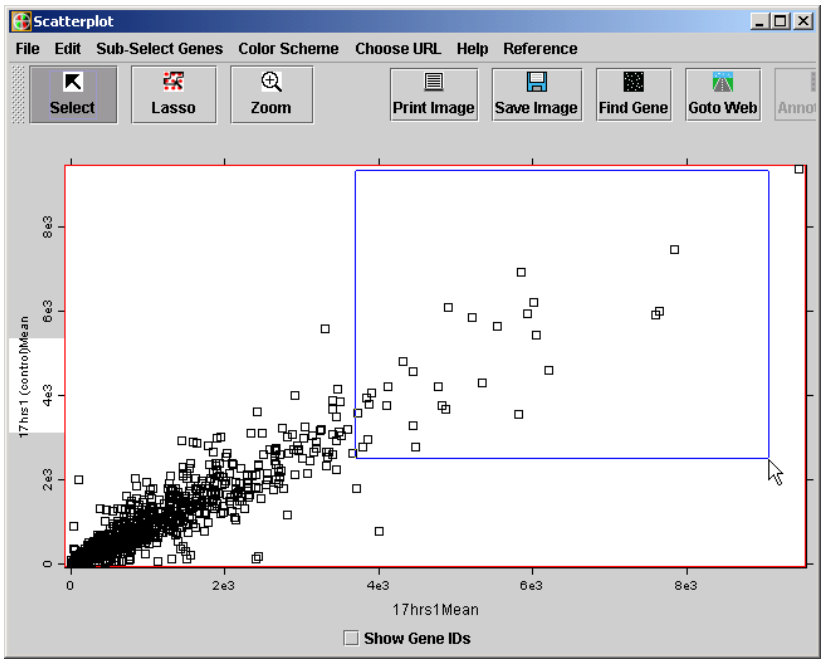


48. Click on while holding the <ctl> key Mean listed under 17hrs1 and 17hrs (control) in the primary GeneSight-Lite User Interface. Be certain both “Mean” labels are highlighted.

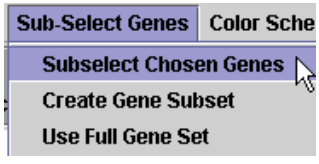


49. Click the Scatter Plot Button from the toolbar.

50. Drag with the mouse to select all the genes located along the top right of the plot, or those genes with high intensity values.

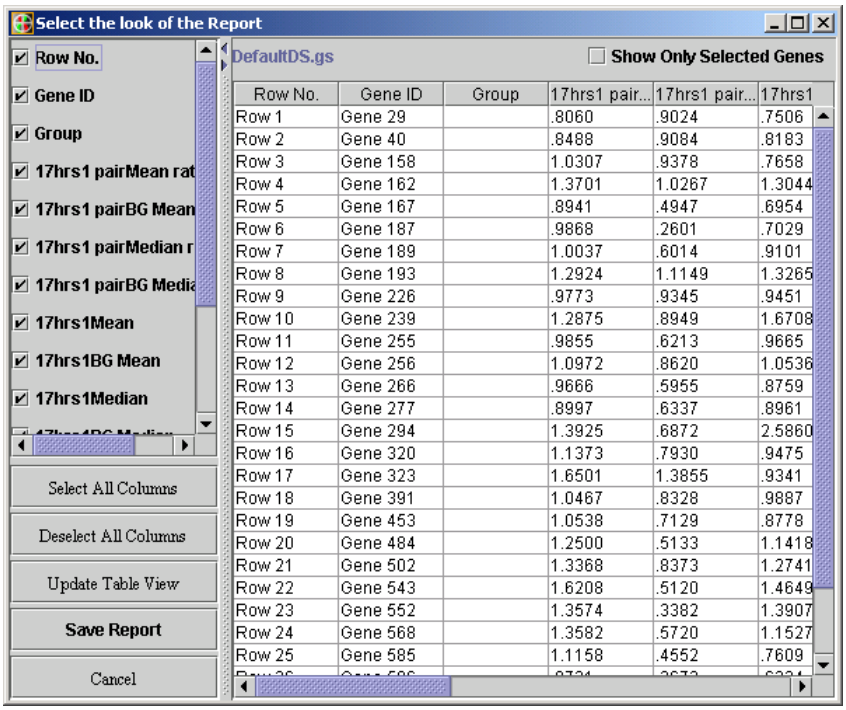


51. From the menu bar, select Sub-Select Genes followed by Sub select Chosen Genes.



‘

52. Enter “Hot Genes” for the name of this sub selection.
53. Close the Scatter Plot Window
54. From the menu bar of GeneSight-Lite, select Utilities then Generate Report.
55. Click Save Report to save the selected genes to a text file on the file system. The file is a common tab delimited text file, which can be opened by programs such as Microsoft WordPad or Excel.



2.2 Premanufactured Array Analysis

ImaGene provides the ability to load templates for many commercially available arrays. A template within ImaGene is the combination of a grid and the genelist, or Gene IDs, required to easily quantify and name the various probes on the array. BioDiscovery provides a selection of these templates to its customers as a service to aid their array analysis. For a complete listing of templates currently available, contact BioDiscovery support. The following Lessons will be covered:

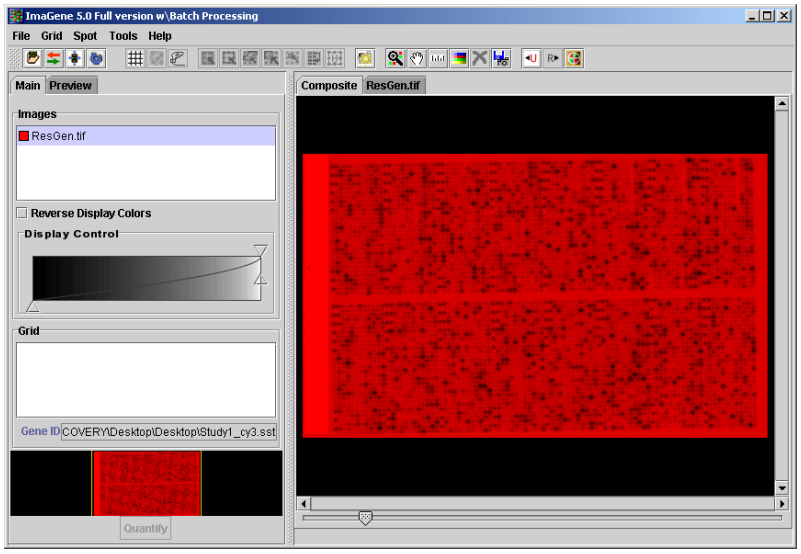
- **Image Selection** - Describes how to load an image into ImaGene and use the controls on the Image tab to enhance the on-screen display of the image.

- **Template Selection** –Demonstrates the loading and placement of BioDiscovery premade templates.

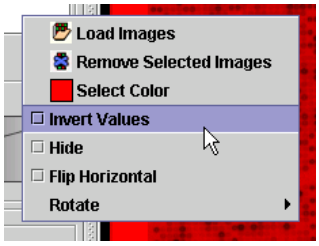
- **Quantification** - Describes the quantification and subsequent output of data.

Image Selection

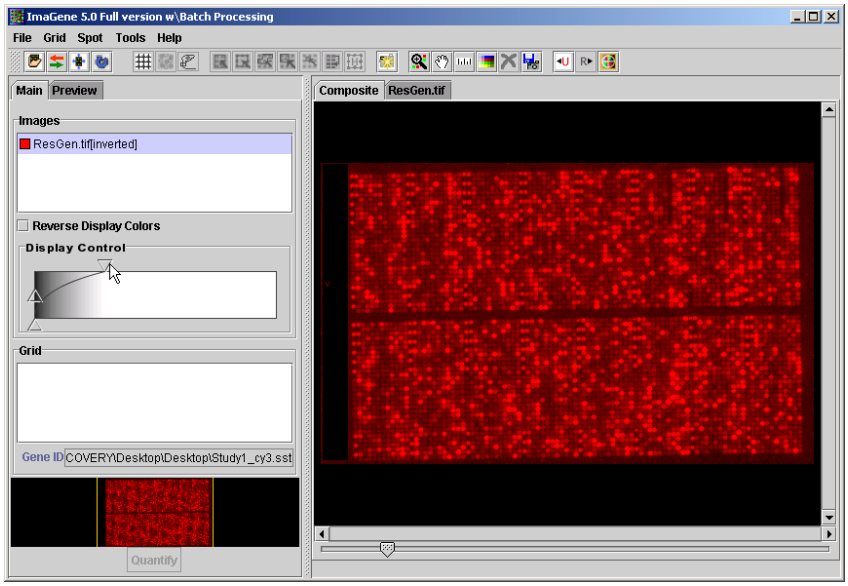
1. Launch ImaGene from the desktop icon of from the ImaGene folder within the Windows Start Menu.
2. From the menu bar, select File followed by Load Images.
3. Browse to the Samples folder within the ImaGene folder. The location is typically, C:\ImaGene\Samples.
4. Select ResGen.tif.
5. Click the Open Button to complete the image loading. A progress bar displays time remaining. This particular image demonstrates the fact that not all images contain the proper information regarding intensity. In this image, the default is that the background contains high intensity values and the signal regions possess low intensity. Since this is not how the image is in the real world, we must Invert the image with ImaGene.



6. Right click on the Images panel and select Invert Values.

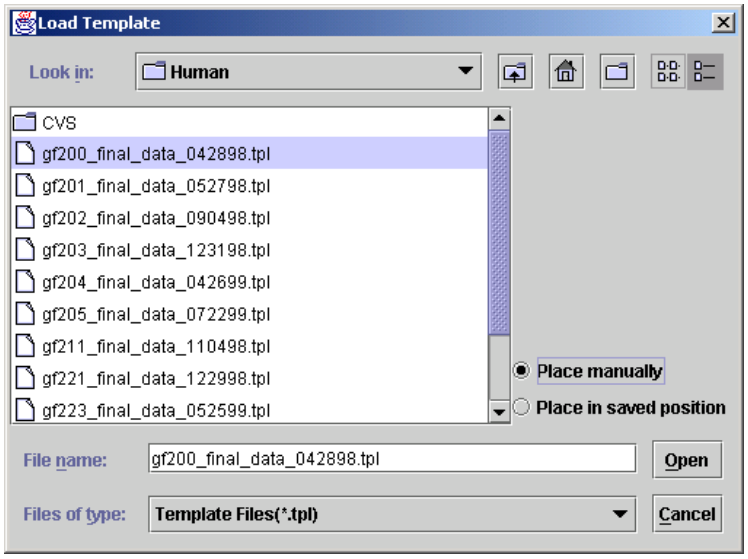


7. Slide the top triangle of the Display Control Panel to the left ¾ of the panel's length. The image should now be clearly visible.

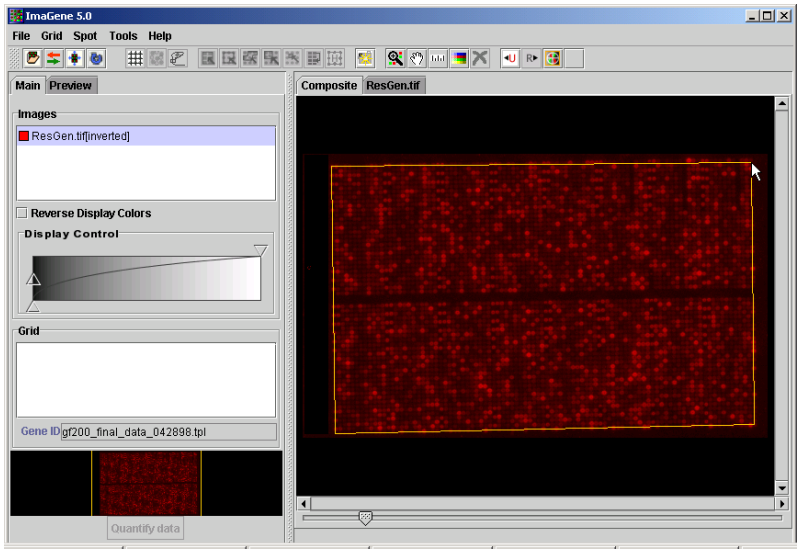


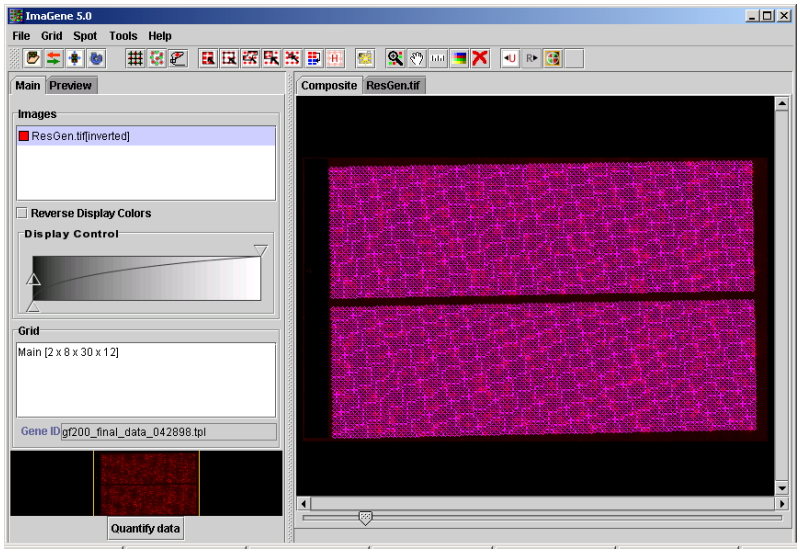
Template Selection

8. From the menu bar, select Grid followed by Load Template.
9. Browse to the Research Genetecs folder, the Human folder and select the gf200 template.



10. Select Place Manually
11. Click the Open Button
12. Click the four corners of the entire array. The grid will be placed exactly on the array taking into account differences in scale and rotation.





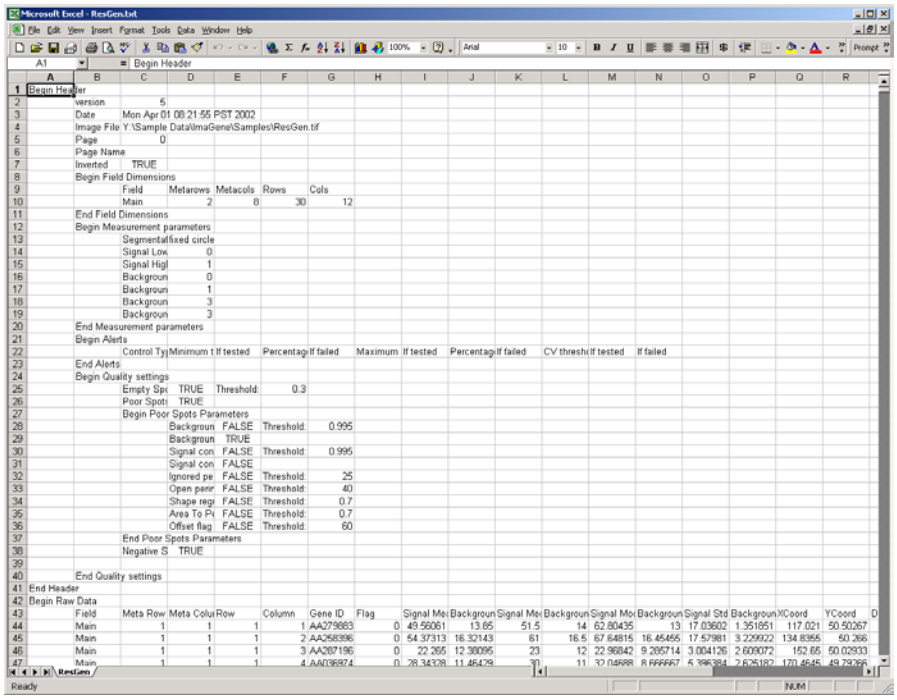
Quantification

13. Click the Quantify Button under the Main Tab.

14. Once the quantification completes, click the Save Button located under the Results Tab.

15. Specify a file name and location to save the data file.

16. Using Windows Explorer or My Computer, navigate to the data file within the file system.
17. Open the file using either MS Excel or Microsoft Wordpad. Notice the GeneID column contains gene names as defined by Research Genetics.



The use of templates through ImaGene provides a convenient and simple way to perform grid placement and associate gene information with clone locations.

2.4 Batch Processing

This tutorial demonstrates how to use batch processing within ImaGene. Batch processing is an additional module which in not included by default with the base ImaGene. Please contact BioDiscovery support with any question regarding eligibility. The lessons learned within this tutorial include:

- **Preparing for batch processing** – Prior to performing batch processing, several files need to be made available to ImaGene.

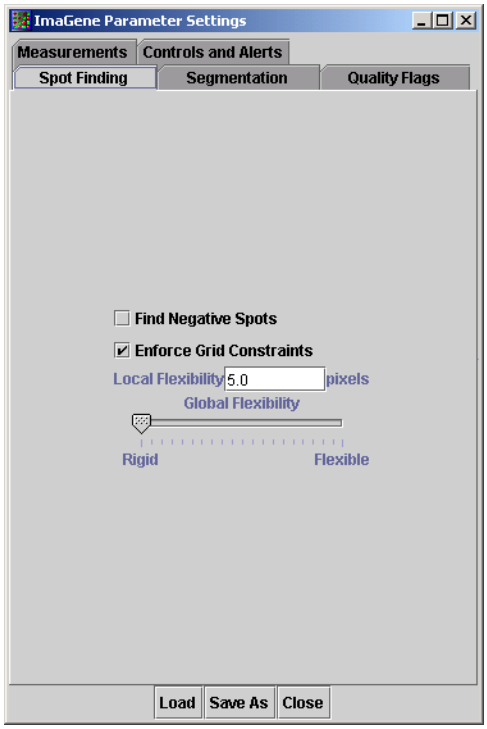
- **Adding Entries to the Batch** – Entries are the images and the corresponding settings to be used when performing batch processing

- **Post Processing** – Start times, stop times and any errors which occurred during the batch are recorded to a simple text file for later analysis. Like wise, results can be reviewed to determine processing quality.

2.4.1 Preparing for batch Processing

Prior to performing batch processing a configuration file and either a grid or a template file must exist. To generate these files, perform the following:

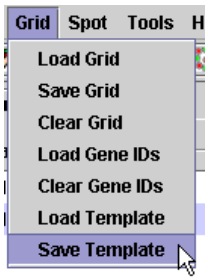
1. Launch ImaGene if the software is not already started
2. Load a sample image which is indicative of the group of image to be batched. In this case, load Study1_cy3.TIF from the ImaGene samples directory.
3. The next step is to setup the configuration to be used. To do this, open the ImaGene Parameter Settings Window by selecting File followed by settings from the menu bar. For this example, be certain AutoSegmentation is checked, located under the segmentation tab, and that all the measurements are checked, located under the measurements tab. Go to Quality Flags tab, check Empty and Poor spots, set empty spot threshold to 1. Click Change Parameters button and click Default on Quality Parameters dialog, close dialog. Finally, click the Save As button to save the configuration file to the file system to a location you choose. Remember this location as we will recall it shortly.



4. Click the Close Button to exit the ImaGene Parameters Settings Window
5. The next step is to perform grid placement. To do so, right click on the Grid Panel and select Create Grid. Enter 8 for the Rows and 12 for the Columns. The Min and Max Diameters should be set to 9.
6. Click the Place Grid Button and click on the four corners of the array.
7. Specify a gene ID to be used with this array. To do so, from the menu bar, select Grid followed by Load Gene IDs. Locate and select the study Gene IDs within

the ImaGene samples directory. Note that the Gene ID, while helpful, is not required for batch processing.

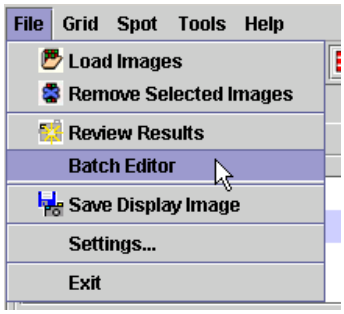
8. The final step is to save a template which can be used when setting up the batch. To save a template, select Grid followed by Save Template from the menu bar. Specify a name and save this to the location of your choice.



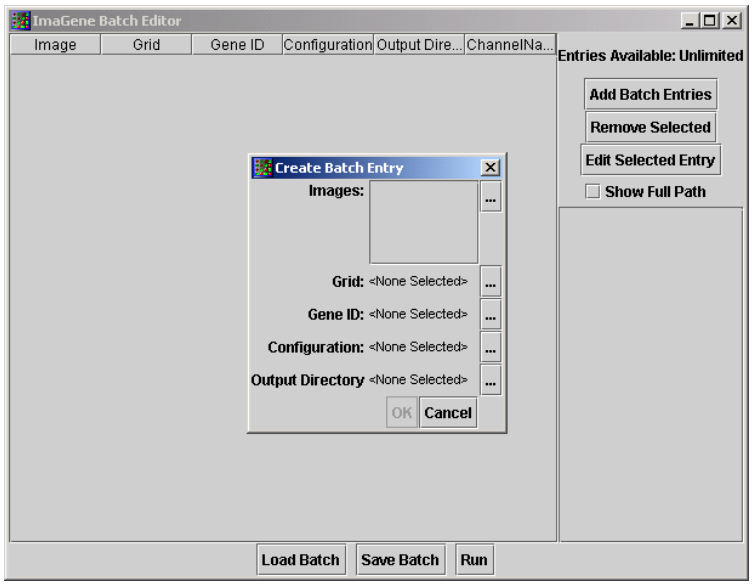
2.4.2 Adding Entries to a Batch

Once the configuration file and the template files exist, these can be combined with 1 or more images to create a batch. To add entries to a batch:

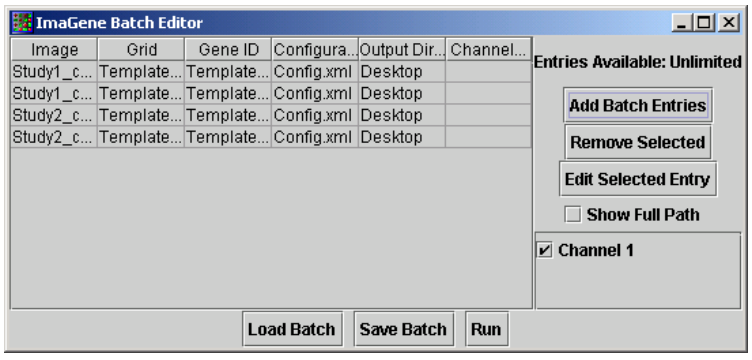
1. From the menu bar, select File followed by Batch Editor.



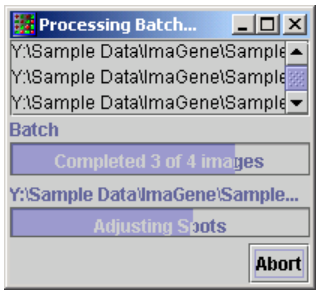
2. Click the Add Batch Entries Button on the Batch Editor Window.
3. The Create Batch Entry window appears allowing us to specify the files previously created as well as other information. First click the images button and browse to the ImaGene samples directory. Select all four image files that begin with “Study”. There should be four files. Holding the <ctr> key while selecting the images allows several to be selected at once.



4. Next click the Gird Button. Either a grid or a template may be specified here. Browse to and select the template we created previously.
5. Notice the Gene ID selection below the Grid is now not enabled. This is because we previously specified a gene ID and associated it with this template, hence there is no need to do so again.
6. Click the Configuration button and specify the configuration previously created.
7. The last parameter to specify is the output directory. ImaGene needs to know where to store the quantified data files as well as the snapshot, or sst, files.
8. Click the OK Button to add the entries to the batch. Notice within the Main Batch Editor Window there are four entries corresponding to the four images previously specified. If another set of images are to be batched, this process of adding entries can be repeated as many times as needed.



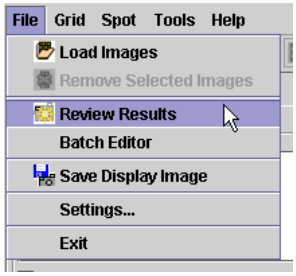
9. Click the Save Batch Button to save this batch for possible reuse later. From the Save As window, specify a location and file name for the batch file.
10. Finally, click the Run Button to begin the batch. A small Batch Processing Window appears displaying both the progress through the current image and the progress through the entire batch. Upon completion of the batch, this small window simply closes.



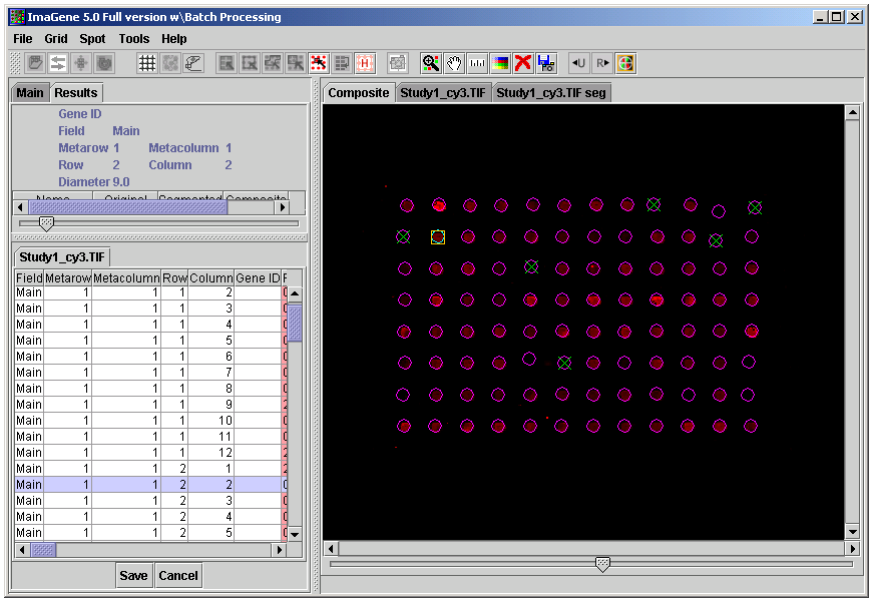
2.4.3 Post Batch Processing

Ultimately, the utility and convenience batch processing provides means very little without accurate quantification. To view the results of Batch Processing, ImaGene includes two tools, the results reviewer and the ImaGene log file.

1. Launch the ImaGene software.
2. From the File Menu, select Review Results.



3. Browse to and select any one of the four snapshot files, or files ending with an sst extension, which were produced during the previous batch processing. The file will be located within the same directory as the data output. Click the Open Button.
4. ImaGene loads the images, data, quality measures, and flagging as occurred though batch processing. From here, you are free to determine the quality of the batch processing and whether or not the image needs to be manually processed.



5. Also located within the data output directory is a file called Log.txt. This file contains start and stop times for all the batches. It also contains any errors that may have occurred.

Begin Batch Mon January 01 08:02:50 PST 2002
Begin Run
Info: current image: Y:\Sample Data\ImaGene\Samples\Study1_cy3.TIF
Info: channel name:
End Run
Begin Run
Info: current image: Y:\Sample Data\ImaGene\Samples\Study1_cy5.TIF
Info: channel name:
End Run
Begin Run
Info: current image: Y:\Sample Data\ImaGene\Samples\Study2_cy3.TIF
Info: channel name:
End Run
Begin Run
Info: current image: Y:\Sample Data\ImaGene\Samples\Study2_cy5.TIF
Info: channel name:
End Run
End Batch Mon Apr 01 08:02:58 PST 2002

Batch processing is an easy and flexible way to provide image analysis in a high throughput environment, or walk away convenience for overnight processing. The previous example demonstrates the steps required to perform at batch. The overall success of the batch depends on a number of factors including the quality of the images, the accuracy of the settings and the uniformity of the images to one another.

Part 3: GeneSight-Lite

3.1 What is GeneSight-Lite?

After intensity measurements are extracted from an image, there is a need for further data processing. ImaGene incorporates a powerful tool that provides creating simple types of experimental design, data cleansing, transformation, normalization, visualization etc. This tool is called GeneSight-Lite and can be launched at any time using corresponding button on the toolbar. Please, be patient while it launches (might take up to 20 sec for some machines).

GeneSight-Lite will start with data set builder GUI, where you can build your data set for further analysis. Data set may include data from multiple images you processed using ImaGene. To build data set you will have to arrange ImaGene output files (tab-delimited text files) into a specific experimental design.

Once data set is built, you can refer to Data Preparation tool for data cleansing, transformation and normalization. After all necessary preprocessing is applied to the data, you can start using various visualization and analysis tools of GeneSight-Lite. Aforementioned tools include:

- Histogram
- GenePie tool for easy comparison of gene expressions across different experiments
- Scatter Plot (2- and 3-dimensional)
- Partition Manager
- Hierarchical Clustering (2 linkage methods and 5 distance measures)
- Interface with web based public genetic data
- Time series visualization

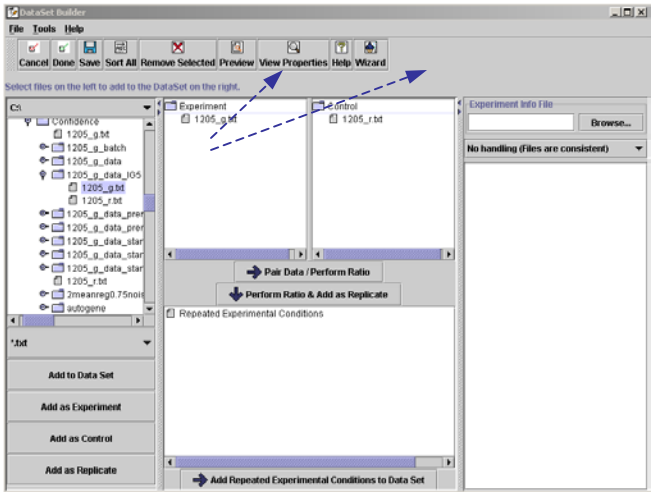
3.2 How to use GeneSight-Lite

For details on GeneSight-Lite usage please refer to GeneSight-Lite user's manual included in ImaGene distribution package. In this section we will provide some guidelines for using basic visualization tools.

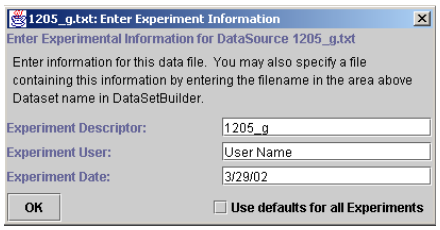
- **Scatter plot.** To build a scatter plot on a data you just quantified you need to follow these steps:

1. Make sure that you saved quantification results from current image (clicked Save button at the bottom of quantification table).
2. Click GeneSight-Lite button on the toolbar . And wait until GeneSight-Lite GUI pops up.

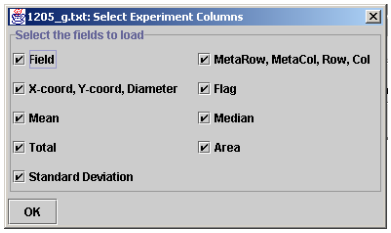
3. GeneSight-Lite will start with a DataSet Builder window. You can either use its Wizard or construct the data set using Builder window directly. We will explain how to use the latter. First find result files saved by ImaGene in the file browse window on the left-hand side. Drag the file considered to be control data into Control window, and the file considered to be experiment data into Experiment window.


DataSet Builder
File Tools Help
Cancel Done Save Sort All Remove Selected Preview View Properties Help Wizard
Select files on the left to add to the DataSet on the right.
Experiment
Control
Experiment Info File
Browse...
No handling (Files are consistent)
Pair Data / Perform Ratio
Perform Ratio & Add as Replicate
Repeated Experimental Conditions
Add to Data Set
Add as Experiment
Add as Control
Add as Replicate
Add Repeated Experimental Conditions to Data Set

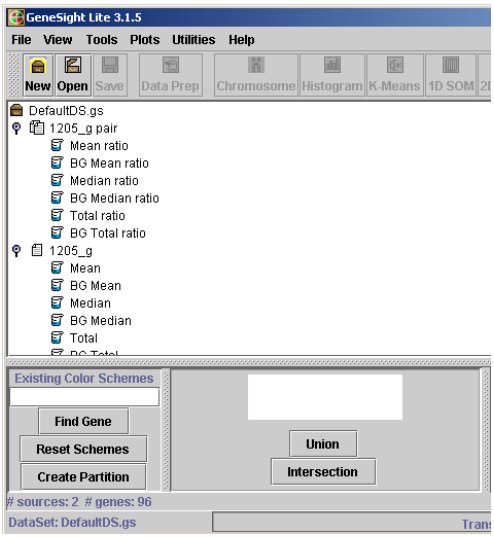
4. Click on "Pair Data/Perform Ratio" button, the new data set will show up in the window on the right-hand side. Click "Done" button on Builder's toolbar. Builder will ask you to enter such information as experiment date and user name to tag the data set.


1205_g.txt: Enter Experiment Information
Enter Experimental Information for DataSource 1205_g.txt
Enter information for this data file. You may also specify a file containing this information by entering the filename in the area above Dataset name in DataSetBuilder.
Experiment Descriptor: 1205_g
Experiment User: User Name
Experiment Date: 3/29/02
OK
Use defaults for all Experiments

Specify the information and click "Ok". Additional dialog will pop up asking you to select measurement that you want to import into GeneSight-Lite.

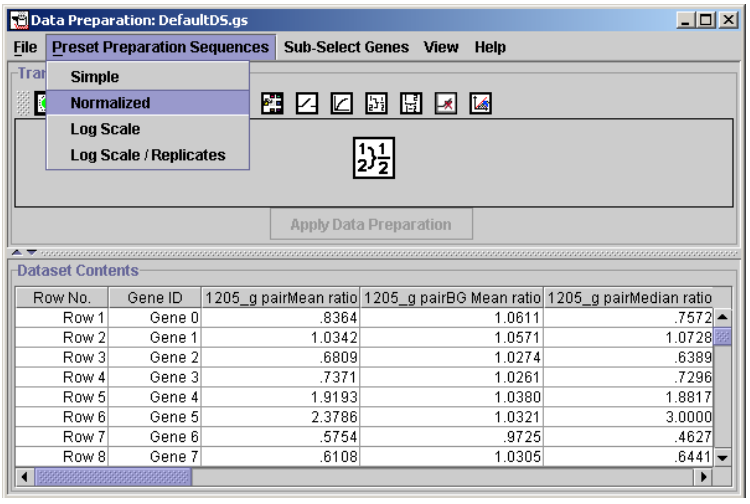


Select measurements and click “Ok”. New data set will be created and corresponding measurements will be shown on the left-hand side of GeneSight-Lite window. For more information on how to build a new data set please refer to GeneSight manual, Chapter 6.



Note: if you have only one file with measurements, drag this file directly into the right-most window of DataSet Builder and hit “Done”.

5. Select the data set and go to Tools menu of GeneSight-Lite. Select Data Preparation tool. It will ask you if you want to use Preview Mode. Answer “No”. The window for Data Preparation tool will pop up. Select one of preset data preparation sequences from Preset Preparation Sequences menu.

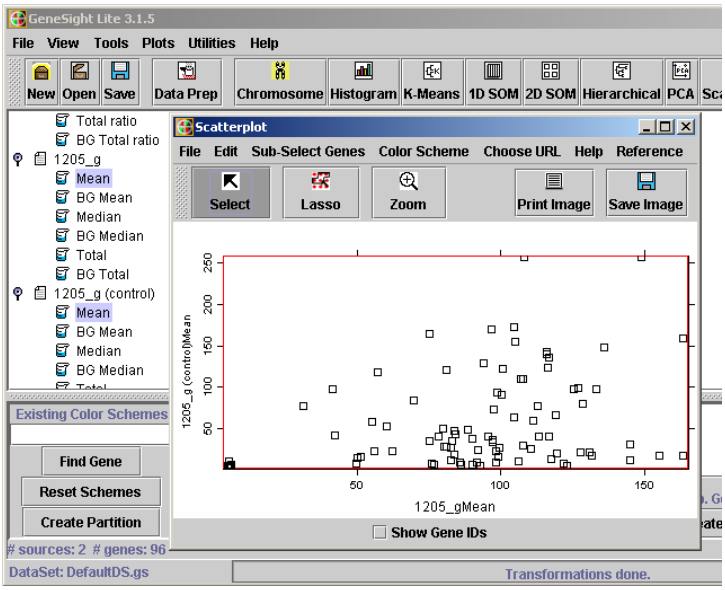

Data Preparation: DefaultDS.gs
File Preset Preparation Sequences Sub-Select Genes View Help
Simple
Normalized
Log Scale
Log Scale / Replicates
Apply Data Preparation
Dataset Contents
Row No. Gene ID 1205_g pairMean ratio 1205_g pairBG Mean ratio 1205_g pairMedian ratio
Row 1 Gene 0 .8364 1.0611 .7572
Row 2 Gene 1 1.0342 1.0571 1.0728
Row 3 Gene 2 .6809 1.0274 .6389
Row 4 Gene 3 .7371 1.0261 .7296
Row 5 Gene 4 1.9193 1.0380 1.8817
Row 6 Gene 5 2.3786 1.0321 3.0000
Row 7 Gene 6 .5754 .9725 .4627
Row 8 Gene 7 .6108 1.0305 .6441

Or alternatively you can create your own sequence by dragging preparation icons into the area of under toolbar, where the sequence will be displayed. Click “Apply Data Preparation” button. Close data preparation window when the preparation

sequence is finished. For more information on how to construct data preparation sequence please refer to GeneSight manual, Chapter 7.

6. Select any pair of measurements in GeneSight-Lite main window.
7. Click “Scatter Plot” button on the toolbar

. 2-D scatter plot window will pop up.



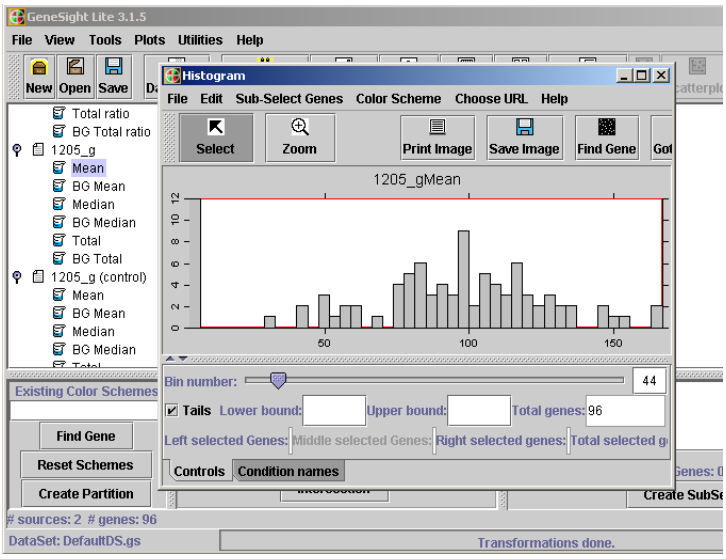
Note: If you select three measurements, 3-D scatter plot will be shown.

8. For manipulations with within Scatter Plot window refer to GeneSight manual, Chapter 9.

- **Histogram**

1. Follow steps 1-4 from the previous section.
2. Select the measurement of interest in GeneSight-Lite main window.
3. Click “Histogram” button on the toolbar

. Histogram window will pop up.

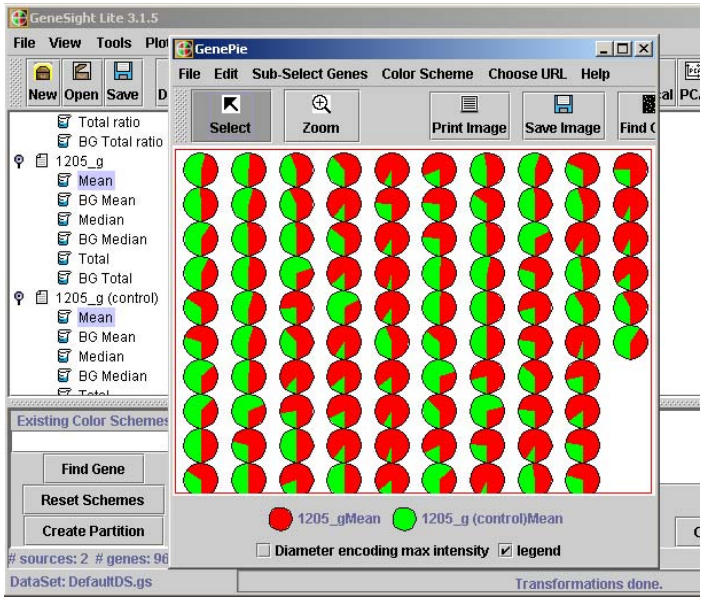


4. For manipulations with within Histogram window refer to GeneSight manual, Chapter 9.

- **Gene Pie**

1. Follow steps 1-4 from the Scatter Plot section.
2. Select any number of measurements of interest in GeneSight-Lite main window.
3. Click “Gene Pie” button on the toolbar

. Gene Pie window will pop up. Gene pie schema shows relative value of every selected measurement within each spot.



4. For manipulations with within Histogram window refer to GeneSight manual, Chapter 9.

- **Go To Web**
  When using any of the tools mentioned above, select a gene or set of genes and click on the "Go To Web" button

. You will be automatically directed to the web based public genetic data.

- **Report**
  GeneSight-Lite also has various ways of generating reports; please refer to GeneSight manual, Chapter 10.

  Note: all GeneSight-Lite tools can be used simultaneously and any gene sub-selection in one tool will propagate into all other tools.

Part 4: Installation and Licensing

Windows

Introduction

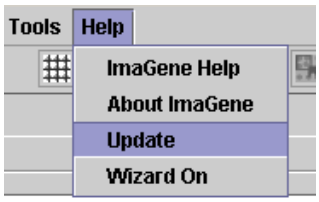
ImaGene uses a standard InstallShield® installation under Windows. Most users are familiar with this easy-to-use format and will not encounter any difficulties. The ImaGene installation simply places the necessary files on the host computer and modifies the Start Menu.

ImaGene Installation

1. If you are installing ImaGene on a Windows NT, 2000, or XP operating system, *you MUST log in with an account that has Administrator access*.
2. Launch the installation by double-clicking on the exe file.
3. Review the version information on this screen, and then click the Continue button to unpack the installation files.
4. When the "Welcome to ImaGene Setup" screen appears, click the Next button.
5. Read the license agreement, and then click the Yes to agree to the terms and conditions.
6. Read the setup notes, and then click the Next button to continue.
7. Review the list of selected components to be installed. Note the default location for the ImaGene program folder. If you do not want to use this location, click the Browse button and select another location. Then click the Next button.
8. Note the default name (ImaGene) of your program folder. This will be the location of the ImaGene shortcuts on your Start Menu. If you do not want to use this name, enter another name in the Program Folders field. Then click the Next button to begin the installation. An installation progress bar appears so that you can monitor the installation.
9. Note that ImaGene requires that Java Runtime Environment (JRE) 1.3 be installed on the computer. During installation, ImaGene determines if the Java 1.3 Runtime Environment (JRE 1.3) needs to be installed. If ImaGene cannot locate the proper runtime version, you will be prompted to install Java before continuing the installation. The ImaGene installation will automatically proceed after Java is installed.
10. When the installation is complete a message box appears. Click the Finish button to complete the installation and close the install program.
11. A webpage now appears with the latest release notes for this particular version of ImaGene. Close the web browser when done reviewing these release notes.
12. Prior to use, please review the next section on licensing ImaGene.

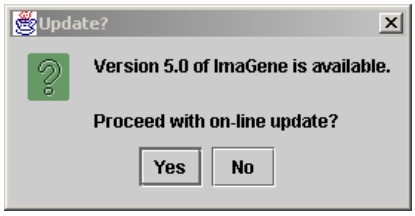
ImaGene Update

If computer is connected to the Internet, ImaGene automatically checks for any update available from BioDiscovery at the start-up. User can control frequency of update checks through menu item Help->Update.



Five different frequencies are available from checking at every start-up to not checking at all.

When ImaGene detects a new version available for download, the following message will be shown.



To proceed with the update press Yes. Your ImaGene will be updated and your old files will be backed up inside ImaGene installation directory. When update is complete, it will prompt you to restart the software.

Un-Installation Instructions

Introduction

This section explains how to remove ImaGene from your computer. For example, if you are no longer using an older version of ImaGene, you can use this procedure to remove it from your computer.

Note: If you have a licensed version of ImaGene, please contact BioDiscovery Technical Support at support@biodiscovery.com before removing any version of ImaGene from your computer as un-installation may affect your licensing.

ImaGene Un-Installation

1. Click Start -> Settings -> Control Panel -> Add/Remove Programs.
2. Click on "ImaGene" to select this program from the Currently Installed Programs list.
3. Click the Change/Remove button to initiate the un-installation process and display the ImaGene confirmation dialog box.
4. Click the Yes button uninstall ImaGene.
   A second ImaGene confirmation dialog box displays when the un-installation completes. Click the OK button to acknowledge that ImaGene has been removed your computer.

Macintosh

Please see the release notes provided with your ImaGene software distribution.

Unix

Please see the release notes provided with your ImaGene software distribution.

4.2 License Management

4.2.1 License Manager

The License Manager is a small software service/daemon that controls which computers can run a particular BioDiscovery product and what features are available in that product. The main purpose of the license manager is to prevent software piracy, but there are additional benefits as well (such as user license tracking, time-limited demos, and advanced user network permission settings).

BioDiscovery has recently switched over to GlobeTrotter's FLEXlm license manager. This popular license manager provides an excellent balance of features, flexibility, and easy-of-use. It is purely software based, so there is no need for any sort of hardware (like a parallel port dongle) and it can handle licenses from multiple companies. If your institution/business is already running FLEXlm-based products, the addition of BioDiscovery licenses will be very easy.

Many specific FLEXlm questions are answered in this online FAQ:
http://www.globetrotter.com/flexlm/lmfaq.shtml
It is not necessary to review this FLEXlm FAQ, since the basic setup of FLEXlm is covered in the accompanying text.

The FLEXlm license manager can be configured in two possible ways: Node-Locked and Floating.

4.2.2 Node-Locked Licenses

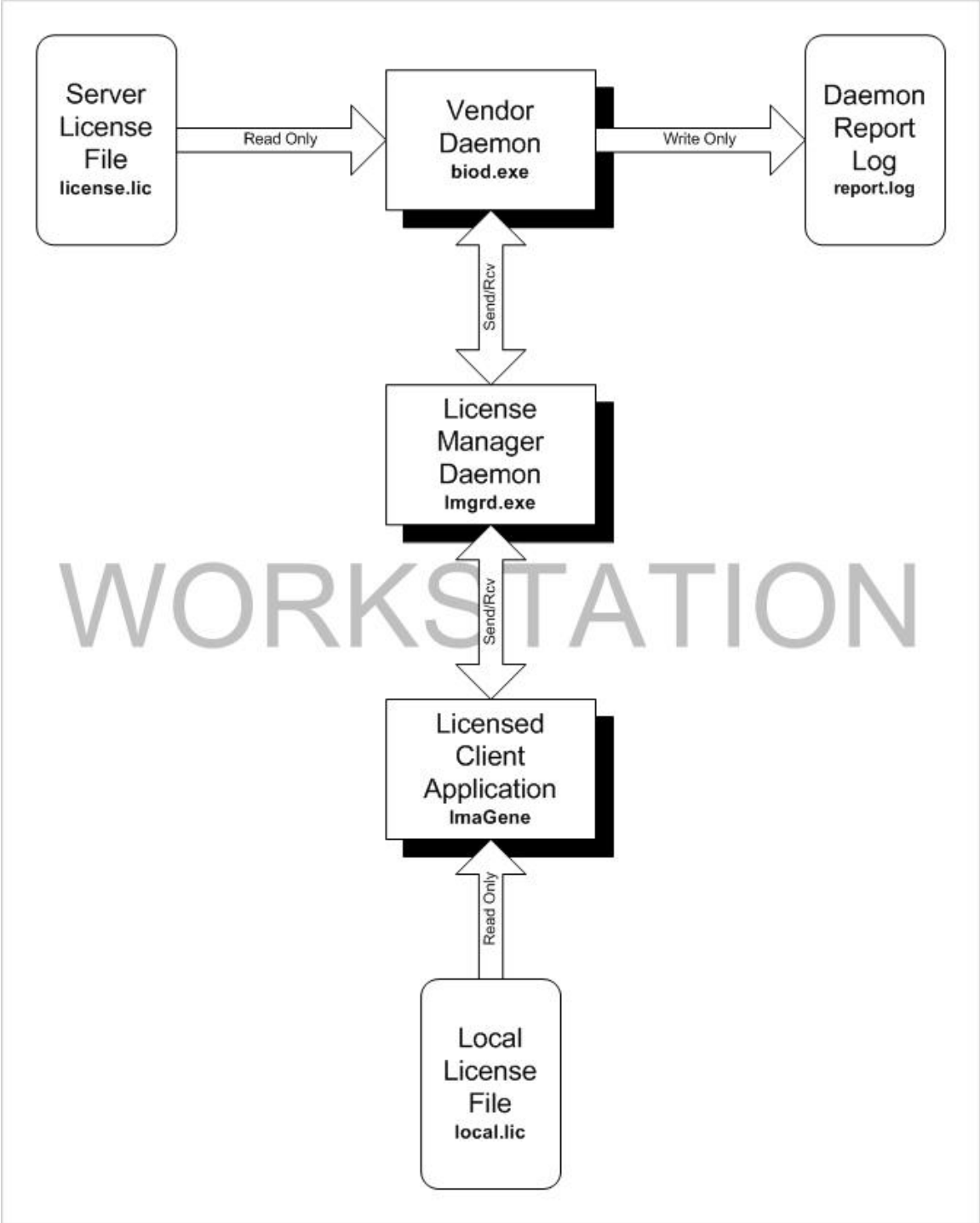
A node-locked license allows BioDiscovery products to run on a single, specific computer. The license is "hard-coded" to that specific computer's ID and will not run on any other computers. Node-Locked is the more common of the two possible configurations.

In order to run in the Node-Locked mode, the computer must be both a FLEXlm license server and it's own client. This requires the user to setup a server on the computer in addition to the regular installation of the software.

The FLEXlm license manager service will run in the background at all times, regardless of whether or not a BioDiscovery product is also running. This may seem inefficient, but the overhead of the license manager is minimal and it would be inconvenient for the user to have to manually start the license manager service every time.

Below is an example of a node-locked computer being both a license server and a client. This diagram is also a good reference since it shows the file names for each part of the license.

FLEXlm Node-Locked Dataflow



WORKSTATION

4.2.3 Floating Licenses

A floating license allows BioDiscovery products to run on a set number of different computers with the limitation that no more than a certain number of computers can run the software simultaneously.

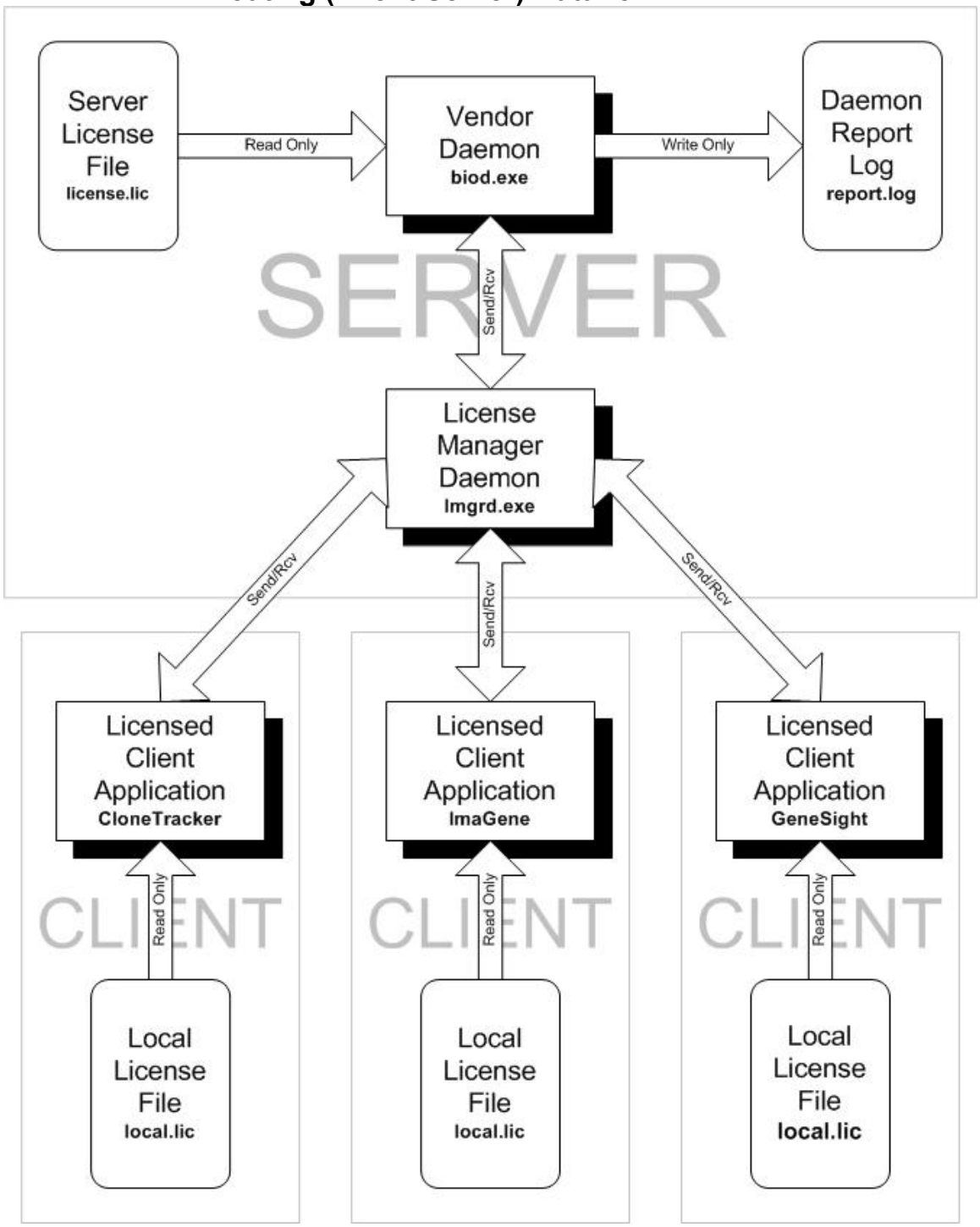
For example, a 5-seat floating license server would allow up to 5 computers access to a particular product. The user may install the software on as many computers as she wishes, but only 5 can run the software at one given time.

FLEXlm provides distinct advantages over other licensing systems in that it can track license usage at the current moment or over an extended period of time. This allows system administrators to track who is checking out licenses most often and if additional licenses need to be purchased.

While less common, the floating license scheme is actually easier to set up than the node-locked scheme since only one server needs to be set up. Each of the client computers only needs to be told where that server is (on a network or even over the Internet).

Below is an example of three clients communicating with a single license server. This diagram is also a good reference since it shows the file names for each part of the license.

FLEXlm Floating (Client/Server) Dataflow



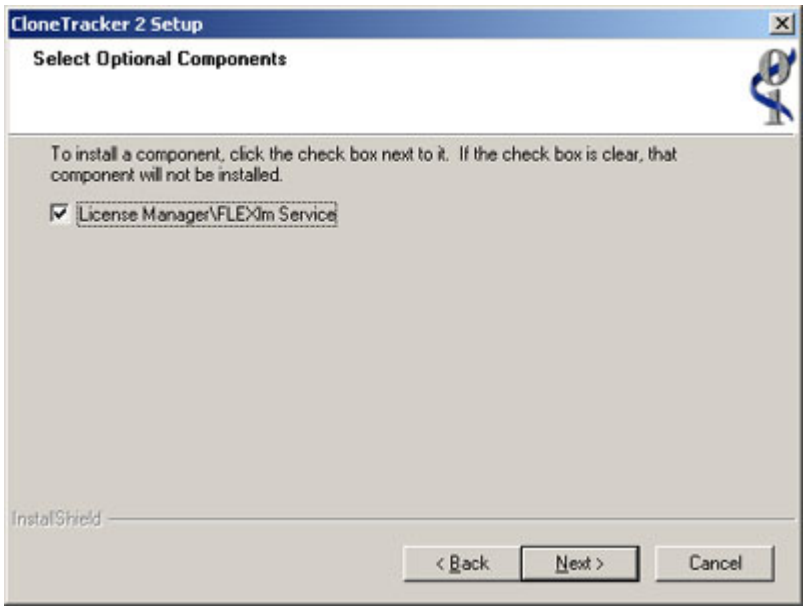
4.3 FLEXlm NodeLocked Installation Guide (for Windows)

These instructions apply to all BioDiscovery products running on any supported platform. In some places the tag {Product} will appear. This represents the name of the BioDiscovery product being installed (like "CloneTracker" for example).

The default install directory for FLEXlm is C:\FLEXlm. In the following examples, it is assumed that this is the directory being used. If FLEXlm is installed into another directory then make the appropriate modifications to the path.

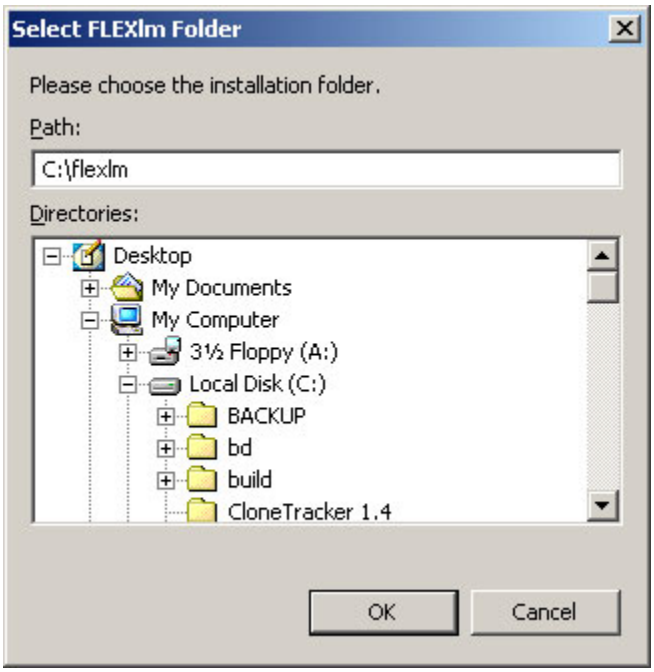
1. Install the BioDiscovery software (with the FLEXlm license manager service)

a. Make sure that the account you are using has administrative privileges on this computer.
b. Make sure that you select the option to install the FLEXlm Service component during the software install.


CloneTracker 2 Setup
Select Optional Components
To install a component, click the check box next to it. If the check box is clear, that component will not be installed.
License Manager\FLEXlm Service
InstallShield
< Back
Next >
Cancel

2. Remember which directory you've selected for FLEXlm in during the install.

a. Note that "C:\flexlm" is the default

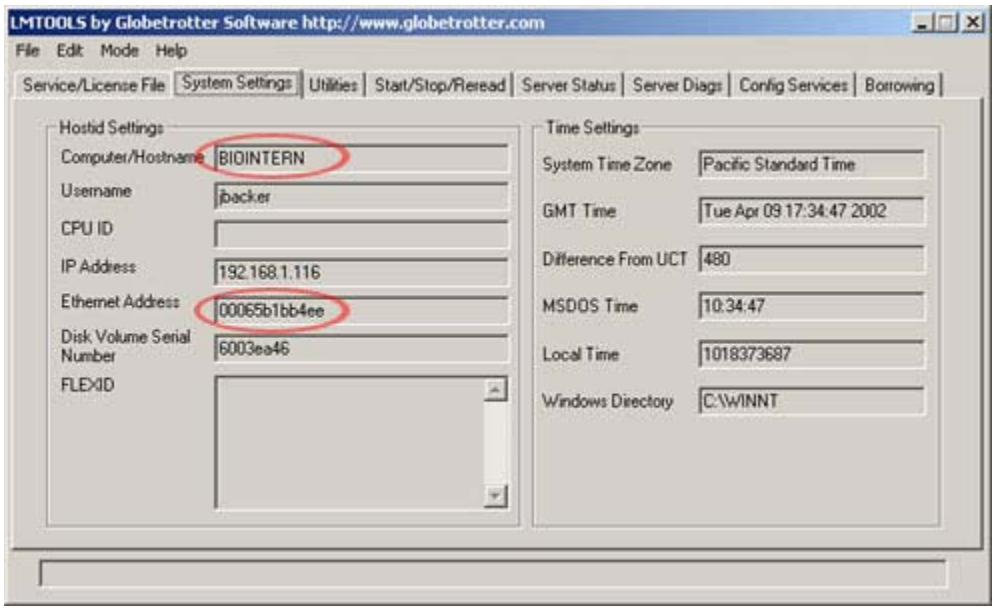


3. Do you already have a license file called "license.lic"?

a. If yes, then skip to step #6.
b. If no, then proceed to step #4.

4. Determine the server's Ethernet Address and HostName.

a. Start the LMTools program by clicking on Start -> Program Files -> {Product} -> LMTools.
b. Click on the System Settings tab.
c. You will see the following screen:



d. The computer's Ethernet Address is used as the computer ID.
e. The Ethernet Address is a 12-digit hexadecimal number. *In this example the address is "00065b1bb4ee".*
f. The computer's Hostname is used to identify the computer on the network. *In this example the hostname is "BIOINTERN".*
g. You can close LMTools by select Exit from the File menu, or clicking the small X button in the top-right corner.

5. Request license file from BioDiscovery.

a. Email support@biodiscovery.com for your customized license file.
b. Please include:
   1. The Computer's Ethernet Address from step #4.
   2. The Computer's Hostname from step #4.
   3. Your name (or the name of who purchased the software)
   4. Your company
   5. Your BioDiscovery Product Serial Number (if you've purchased a copy). You can find your serial number on the case of the CD shipped to you or in the accompanying User Registration form. If you haven't purchased a copy, just list the products you wish to evaluate.

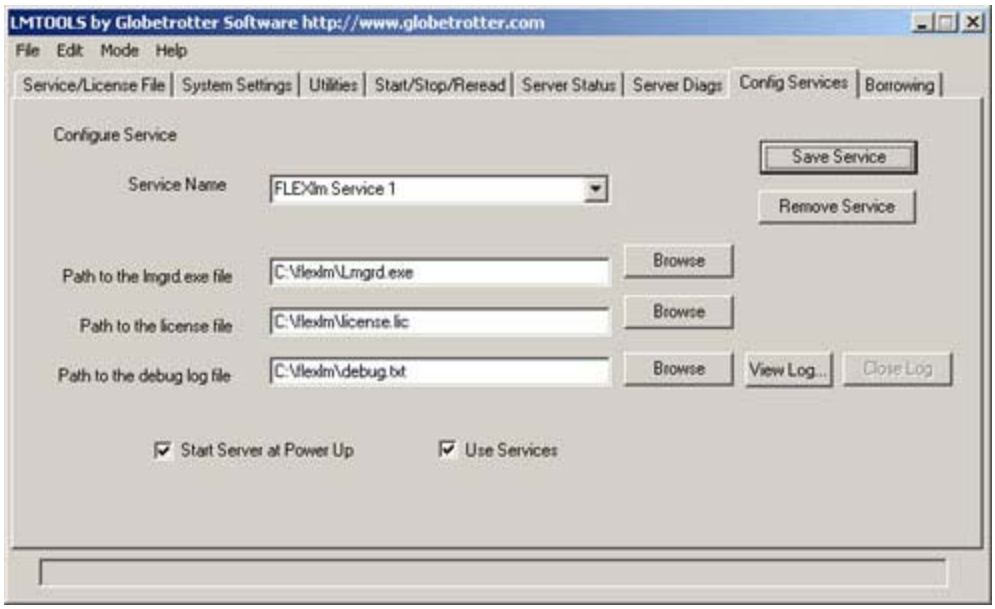
c. BioDiscovery will reply with a small text file called "license.lic". This file contains all of your licensing information.

6. Copy this license file into the FLEXlm directory you specified in step #2.

You should keep a backup copy of this license file on another computer or floppy disk in case of an emergency.

7. Configure the license services

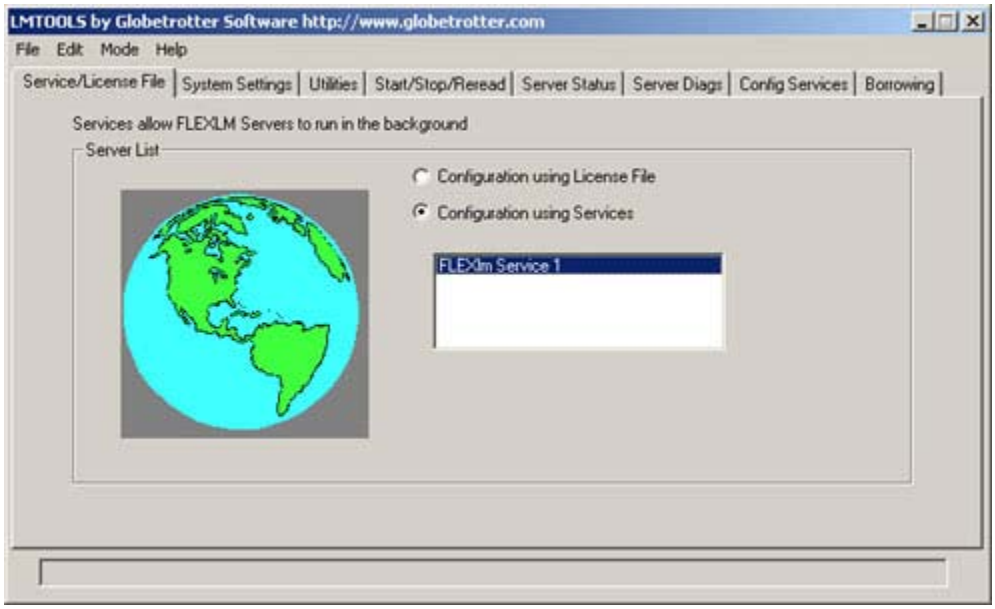
a. Start the LMTools program by double-clicking on lmtools.exe in the FLEXlm directory.
b. Click on the Config Services tab.
c. You will see the following screen:



d. You can leave the Service Name as its default: "FLEXlm Service 1".
e. Enter the Path to the lmgrd.exe file or click the Browse button to the right. In this example it is "C:\flexlm\lmgrd.exe".
f. Enter the Path to the license file or click the Browse button to the right. In this example it is "C:\flexlm\license.lic".
g. Enter the Path to the debug log file or click the Browse button to the right. In this example it is "C:\flexlm\debug.txt".
h. Make sure you place a check mark in both Start Server at Power Up and Use Services. *Note that the Use Services checkbox may be disabled under Windows 95/98/ME.*
i. Click on the Save Service button.
j. Answer "Yes" on the dialog that appears to confirm these settings.

8. Select the license services

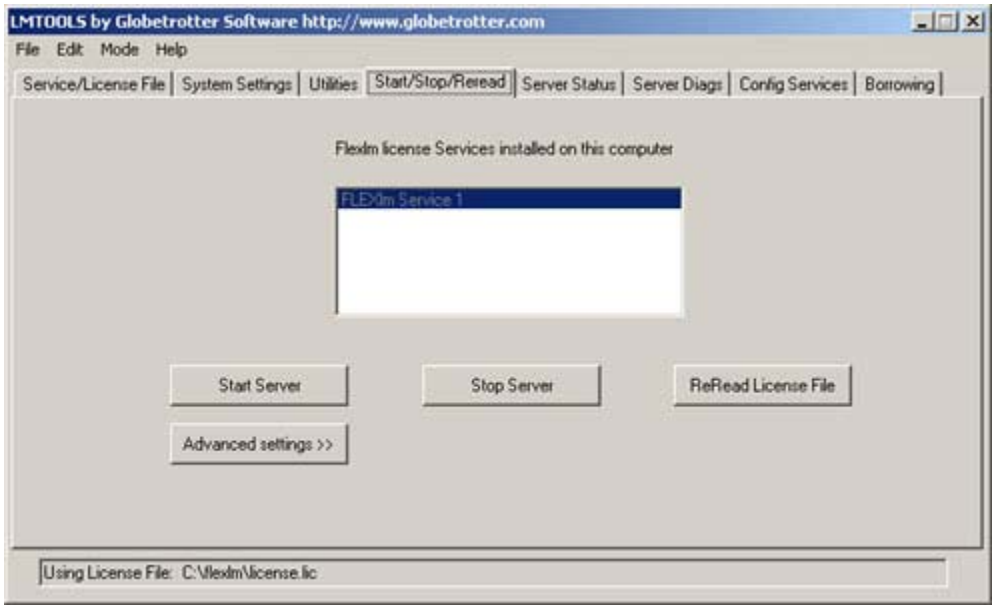
a. Click on the Service/License File tab.
b. The following screen will appear:



c. Select the Configuration using Services radio button.
d. Select the Service Name you used from step #7.

9. Start the License Server

a. Click on the Start/Stop/Reread tab.
b. The following screen will appear:



c. Double-check that the Service Name selected is the same as the one you configured in step #7.
d. Click on the Start Server button.
e. The text at the bottom of the window should change from "Using License File:" to "Server Start Successful."

9. Did the license server start successfully?

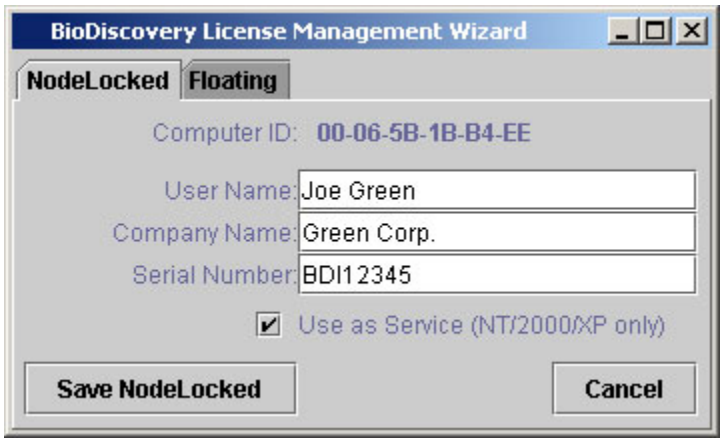
a. If yes, then you're almost done. Skip to step #11.
b. If no, then you need to do some troubleshooting. Proceed to step #10.

10. Troubleshooting the FLEXlm license server

a. Double-check that you entered the correct file paths in step #7.
b. View the license file by right clicking on the "license.lic" in the FLEXlm directory and selecting Open With…
c. Select "Notepad" from the list.
d. Place a checkmark next to "Always use this program to open these files".
e. Click the OK button.
f. The text file will contain a line that looks similar to this:
g. SERVER HOSTNAME 00065b1bb4ee
h. Make sure that HOSTNAME is your computer's Hostname from step #4.
i. Make sure that the following Ethernet Address matches your computer's Ethernet address from step #4.
   If it still doesn't start, then email the C:\FLEXlm\debug.txt file to support@biodiscovery.com

11. Run the License Wizard (configure the local client)

a. Start the License Wizard by clicking on Start -> Program Files -> {Product} -> License Wizard or by double-clicking runwiz.bat in your product directory.
b. Click on the NodeLocked tab.
c. You will see the following screen:



d. Enter your User Name, Company Name, and Serial Number.
e. It is important that you enter your actual Serial Number since this information is used to determine eligibility for upgrades using the BioDiscovery Auto-Update feature.
f. The serial number can be found on the case of the CD shipped to you or in the accompanying User Registration form.
g. If you do not have a serial number, because you're only evaluating this product, you can enter "BDI-DEMO".
h. Ignore the Use as Service checkbox.
i. Click the Save NodeLocked button to save this information and exit.

12. Start the BioDiscovery software.

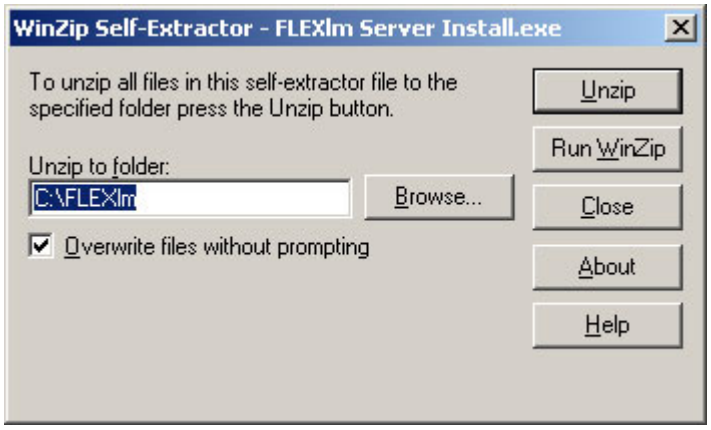
If the license server is running, the software will start up normally.

4.4 FLEXlm Server Installation Guide (for Windows)

The default install directory for FLEXlm is C:\FLEXlm. In the following examples, it is assumed that this is the directory being used. If FLEXlm is installed into another directory then make the appropriate modifications to the path.

1. Extract FLEXlm Server files.

a. Double-click on the self-extracting executable FLEXlm.zip.
b. You will see the following screen:



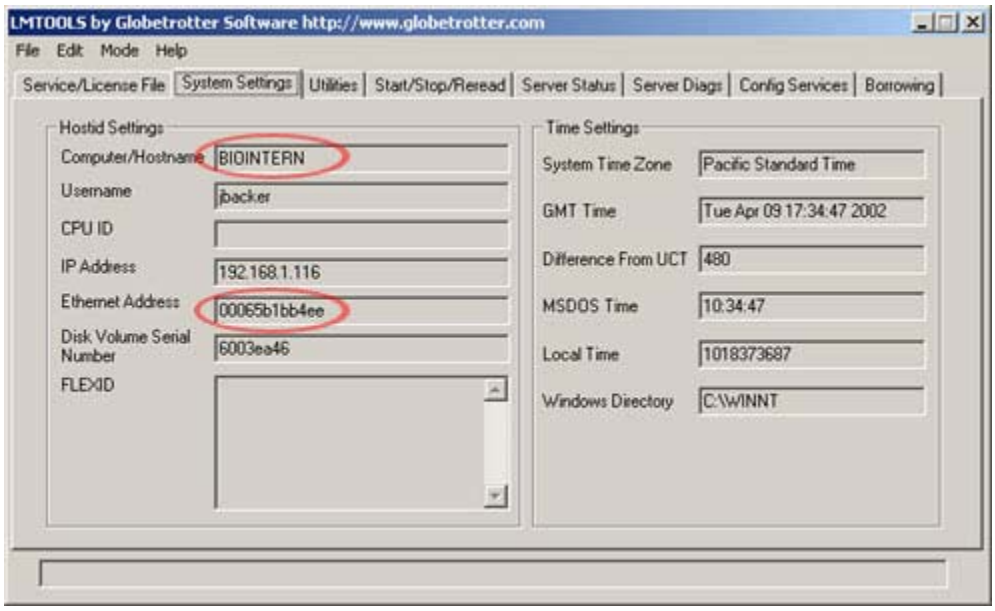
c. Select "Unzip to folder" (C:\FLEXlm by default).
d. Click the Unzip button to extract the files.
e. Click OK once the extraction is complete.
f. Click Close to exit the extraction program

2. Do you already have a license file called "license.lic"?

c. If yes, then skip to step 5.
d. If no, then proceed to step 3.

3. Determine the server's Ethernet Address and HostName.

h. Start the LMTools program by double-clicking on lmtools.exe in the FLEXlm directory.
i. Click on the System Settings tab.
j. You will see the following screen:



k. The computer's Ethernet Address is used as the computer ID.
l. The Ethernet Address is a 12-digit hexadecimal number. *In this example the address is "00065b1bb4ee".*
m. The computer's Hostname is used to identify the computer on the network. *In this example the hostname is "BIOINTERN".*
n. You can close LMTools by select Exit from the File menu, or clicking the small X button in the top-right corner.

4. Request license file from BioDiscovery.

d. Email support@biodiscovery.com for your customized license file.
e. Please include:

2. The Computer's Ethernet Address from step #3.
3. The Computer's Hostname from step #3.
4. Your name (or the name of who purchased the software)
5. Your company
6. Your BioDiscovery Product Serial Number (if you've purchased a copy). You can find your serial number on the case of the CD shipped to you or in the accompanying User Registration form. If you haven't purchased a copy, just list the products you wish to evaluate.

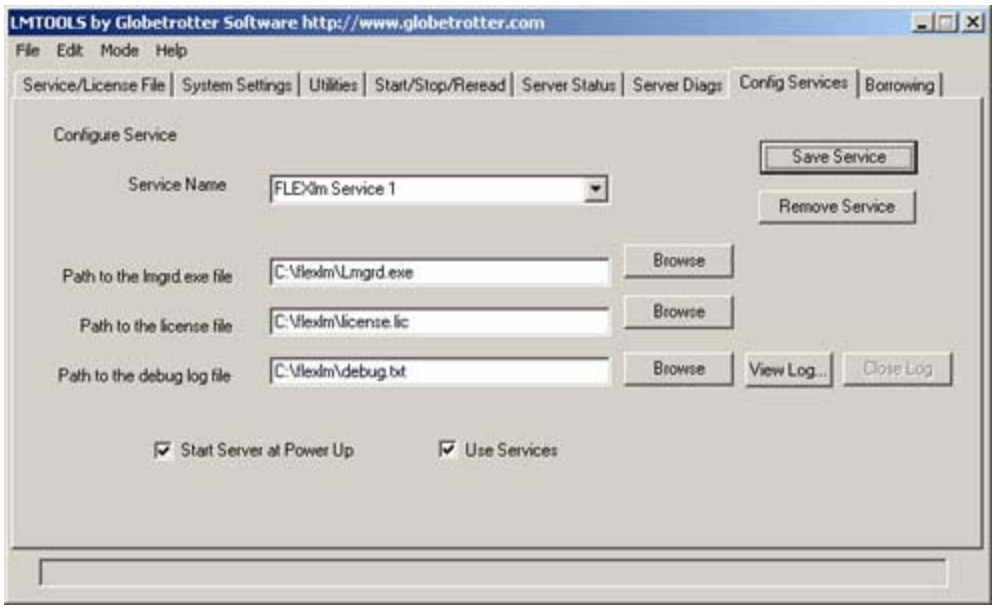
f. BioDiscovery will reply with a small text file called "license.lic". This file contains all of your licensing information.

5. Copy license.lic into your FLEXlm directory.

In truth, the license.lic file can be anywhere on your computer, but we put it in the C:\FLEXlm directory to make it easier to keep track of.

6. Configure the license services

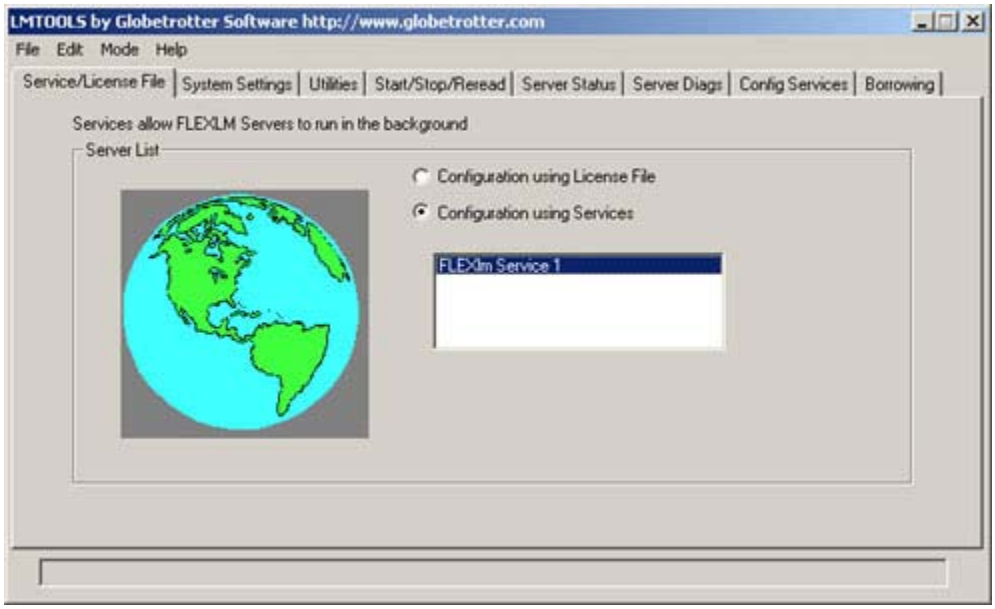
k. Start the LMTools program by double-clicking on lmtools.exe in the FLEXlm directory.
l. Click on the Config Services tab.
m. You will see the following screen:



n. You can leave the Service Name as its default: "FLEXlm Service 1".
o. Enter the Path to the lmgrd.exe file or click the Browse button to the right. In this example it is "C:\flexlm\lmgrd.exe".
p. Enter the Path to the license file or click the Browse button to the right. In this example it is "C:\flexlm\license.lic".
q. Enter the Path to the debug log file or click the Browse button to the right. In this example it is "C:\flexlm\debug.txt".
r. Make sure you place a check mark in Use Services and then Start Server at Power Up. *Note that the Use Services checkbox may be disabled under Windows 95/98/ME.*
s. Click on the Save Service button.
t. Answer "Yes" on the dialog that appears to confirm these settings.

7. Select the license services

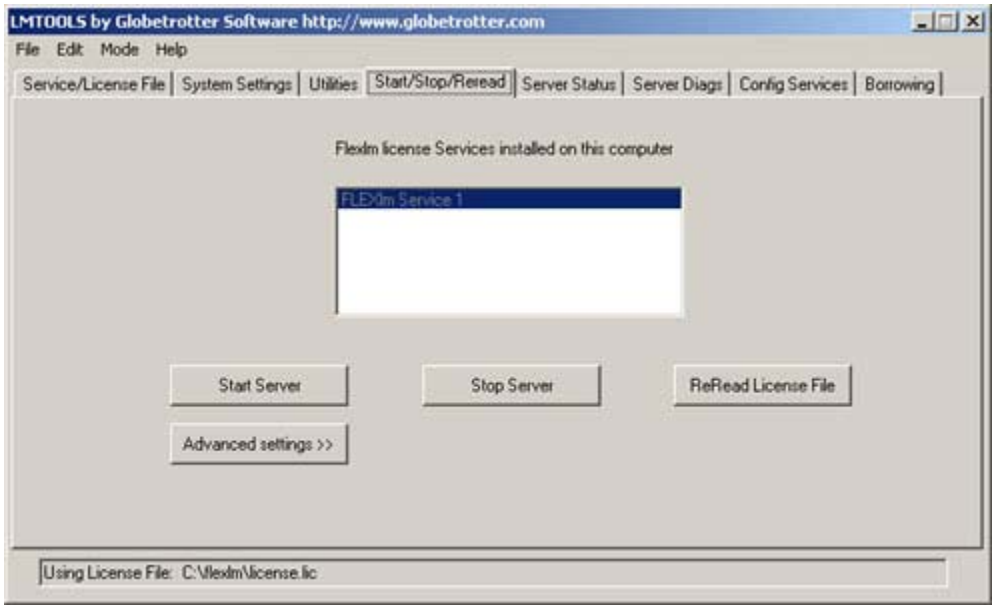
e. Click on the Service/License File tab.
f. The following screen will appear:



g. Select the Configuration using Services radio button.
h. Select the Service Name you used from step #6.

8. Start the License Server

f. Click on the Start/Stop/Reread tab.
g. The following screen will appear:



h. Double-check that the Service Name selected is the same as the one you configured in step #6.
i. Click on the Start Server button.
j. The text at the bottom of the window should change from "Using License File:" to "Server Start Successful."

9. Did the license server start successfully?

c. If yes, then you're almost done. All you need to do now if configure each of the clients to run the software. Please see the FLEXlm Client Installation Guide for details.
d. If no, then you need to do some troubleshooting. Proceed to step 10.

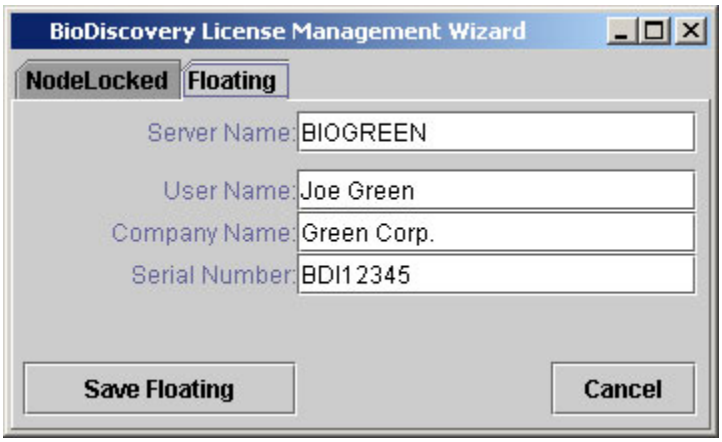
10. Troubleshooting the FLEXlm license server

j. Double-check that you entered the correct file paths in step #6.
k. View the license file by right clicking on the "license.lic" in the FLEXlm directory and selecting Open With…
l. Select "Notepad" from the list.
m. Place a checkmark next to "Always use this program to open these files".
n. Click the OK button.
o. The text file will contain a line that looks similar to this:
p. SERVER HOSTNAME 00065b1bb4ee
q. Make sure that HOSTNAME is your computer's Hostname from step #3.
r. Make sure that the following Ethernet Address matches your computer's Ethernet address from step #3.
s. If it still doesn't start, then email the C:\FLEXlm\debug.txt file to support@biodiscovery.com

4.5 FLEXlm Client Installation Guide (all platforms)

These instructions apply to all BioDiscovery products running on any supported platform. In some places the tag {Product} will appear. This represents the name of the BioDiscovery product being installed (like "CloneTracker" for example).

1. Start the License Wizard by clicking on Start -> Program Files -> {Product} -> License Wizard or by double-clicking runwiz.bat in your product directory.
2. Click on the Floating tab.
3. You will see the following screen:



4. Enter the name of the computer running the license server:
    a. This can be the computer's network name (like BIOGREEN).
    b. This can be the TCP/IP address of the computer (like 192.168.1.6).
    c. This can be an Internet domain name (like www.biodiscovery.com).
    d. This can be this local computer. Use "THIS_HOST" to indicate that this is the very computer serving the license files.

5. Enter your User Name, Company Name, and Serial Number.
6. It is important that you enter your actual Serial Number since this information is used to determine eligibility for upgrades using the BioDiscovery Auto-Update feature.
7. The serial number can be found on the case of the CD shipped to you or in the accompanying User Registration form.
8. If you do not have a serial number, because you're only evaluating this product, you can enter "BDI-DEMO".
9. Click the Save Floating button to save this information and exit.
10. Start the BioDiscovery software. If the license server is running, the software will start up normally.

Part 5: Appendices

5.1 File Specifications

While ImaGene is designed to be flexible with regards to information imported and exported, you must adhere to certain standards regarding file formats.

5.1.1 Gene ID File

The Gene ID file allows you to track information about the genetic material spotted at each location within the array. This information will be saved along with the quantified values in the text output file and visualization tools.

For example, you will be able to click on a GenePie from within GeneSight-Lite and see the specific Gene ID associated with the selected spot in addition to all the other detailed quantification information. To accomplish this, ImaGene needs the Gene ID file to associate a reference ID to each spot in the array of spots in the image.

ImaGene supports two formats for the Gene ID file. The first format will be familiar to existing ImaGene users since the basic structure is the same between versions. The second format is based upon the new multi-level grid structure available in ImaGene. This new format takes advantage of the use of fields, or unique grid/meta grid structures. To deal with the unlimited number of possible fields, and grid structures, an additional column needs to be added to the Gene ID file.

Note: Choose the Gene ID format based on your array structure and preferences. However, when creating the Gene ID file, do not combine the two formats in the same file, as ImaGene will not be able to process the hybrid file format.

Gene ID Format #1 (Traditional Format)

Listed below is a brief description of the various parts of the Gene ID file as well as the precise syntax.

Header - Anything other than the Gene ID information located at the beginning of the file. If you want to include header information, you must start each line with a percentage sign, "%." The percentage sign is an indication to ImaGene not to read this line as gene information but rather continue until a line is encountered matching the structure found below.

Columns - Metarow Number (MR) / Metacolumn Number (MC) / Subgrid Row Number (SR)/ Subgrid Column Number (SC) / Gene ID. Separator: You must use tabs to separate the fields in each row.
Order - ImaGene does not care about the order in which the rows are sorted. It's also not necessary to specify Gene ID information for all the spots in the array.
Gene ID - The Gene ID field can be any alpha-numeric string (including blank spaces) following the Subgrid Column Number. You can specify a database entry followed by the gene name as one option.
Comment Lines - Use the percentage sign, "%," to indicate comment lines.
Creating the Gene ID File - You can use any text processing program, spreadsheet, or database program to generate the Gene ID file. You can also use the CloneTracker software tool to generate this file. CloneTracker manages the information about cDNA plates and the mapping of plates onto the slide. It can also generate the necessary gene ID file to be used by ImaGene.

Insert the accession number followed by a colon and then insert the Gene ID name. For example, all of the following are valid formats for the Gene ID File:

Header information
MR MC SR SC Gene ID
MR MC SR SC Ascension #
MR MC SR SC Ascension #: Gene ID

Note: The actual accession numbers are not provided nor determined by ImaGene or BioDiscovery. The information must either be located by you or provided by the microarray chip manufacturer.

Gene ID Format #2 (ImaGene Format)

The second format follows the same rules as the first except for the addition of a field column:

Columns - Field/Metarow Number (MR) / Metacolumn Number (MC) / Subgrid Row Number (SR)/ Subgrid Column Number (SC) / Gene ID.

Header Information
Field MR MC SR SC Gene ID
Field MR MC SR SC Ascension #
Field MR MC SR SC Ascension #: Gene ID

5.1.2 Output File

ImaGene exports standard tab delimited text files when saving data. The information below is intended to describe the general format of the test file and

explain particular fields therein. ImaGene saves the quantified data from each image processed into individual text files. Due to the multi-image processing capability, ImaGene no longer exports two channel ratio data. Researchers are required to either generate any desired ratio information themselves or import the raw data into a data analysis program such as the GeneSight software tool. The data file is divided into two main parts. The first part is header information about the parameters set for analysis. The second part of the file includes the data extracted from the images.

Note: Due to the nature of tab delimited text files, wrapping of columns is common. To easily view information, BioDiscovery recommends importing the data files into a spreadsheet program such as Microsoft Excel.

```
Begin Header
	version	5.0
	Date	Tue Mar 26 09:19:34 PST 2002
	Image File	C:\BioDiscovery\ImaGene\Samples\1205_g.TIF
	Page	0
	Page Name
	Inverted	false
	Begin Field Dimensions
		Field	Metarows	Metacols	Rows	Cols
		A	1	1	8	12
	End Field Dimensions
	Begin Measurement parameters
		Segmentation Method	auto
		Signal Low	0.0
		Signal High	0.0
		Background Low	0.0
		Background High	0.0
		Background Buffer	3.0
		Background Width	3.0
	End Measurement parameters
	Begin Alerts
		Control Type	Minimum threshold	If tested	Percentage allowed	If failed
	Maximum threshold	If tested	Percentage allowed	If failed	CV threshold	If tested
	If failed
		hot	45000.0	false	0.0%	false	65000.0	false	0.0%	false
	1.0	false	false
	End Alerts
	Begin Quality settings
		Empty Spots	true	Threshold:	0.3
		Poor Spots	true
		Begin Poor Spots Parameters
			Background contamination flag	true	Threshold:	0.9995
			Background tested against subgrid data only	true
			Signal contamination flag	false	Threshold:	0.9995
			Signal contamination test connected to background contamination threshold
	false
			Ignored percentage flag	true	Threshold:	25.0
			Open perimeter flag	true	Threshold:	25.0
			Shape regularity flag	true	Threshold:	0.65
			Area To Perimeter Ratio flag	false	Threshold:	0.65
			Offset flag	true	Threshold:	60.0
		End Poor Spots Parameters
		Negative Spots	true

	End Quality settings
End Header
Begin Raw Data
```

Field	Meta Row	Meta Column	Row	Column	Gene ID	Flag	Signal Mean
Background Mean	Signal Median	Background Median		Signal Mode			
Background Mode	Signal Area	Background Area	Signal Total		Background Total		
Signal Stdev	Background Stdev	Shape Regularity	Ignored Area		Spot Area	Ignored Median	
Area To Perimeter	Open Perimeter	XCoord	YCoord	Diameter	Position offset		Offset X
Offset Y	Expected X	Expected Y	CM-X	CM-Y	CM Offset		CM Offset-X
CM Offset-Y	Min Diam	Max Diam	Control	Failed Control			
Background contamination present	Signal contamination present	Ignored	% failed	Open perimeter failed	Shape regularity failed	Perim-to-area failed	Offset failed
Empty spot	Negative spot						
A	1	1	1	1	0	109.89393939393939	
4.926565874730022	129.5	5.0	132.9090909090909	4.738562091503268	66.0	463.0	
7253.0	2281.0	55.13623039946595	2.9050872336804874	0.584070796460177	0.0	66.0	
NaN	1.0	0.0	33.427956043956044	21.692307692307704	9.0	0.3435990182607777	
0.3298958928437372	-0.09606760762402544		33.09806015111231	21.78837529993173			
33.984848484848484	21.151515151515152	1.0917803796972039	0.8867883337361775	-0.6368601484165772	8.319720247072127	10.339058029748454	
0	0	0	0				
0	1	0	0	0	0		
A	1	1	1	2	0	163.88571428571427	
4.821350762527233	228.5	5.0	248.61111111111111	4.571428571428571	70.0	459.0	
11472.0	2213.0	102.5569587883649	1.2165800511090508	0.6481481481481481	0.0	70.0	
NaN	0.9773843811168245	0.0	55.77061338661339	21.72727272727274	9.0		
0.2811541783968894	0.269943687738035	-0.07860074732736422		55.500669698875356			
21.805873474600105	56.0	21.514285714285716	0.5782336652136307	0.49933030112464394			
-0.29158776031438904		8.236983309361506	11.051234910005755		0	0	
0	0	0	1	0	0	0	0
A	1	1	1	3	0	105.26470588235294	
4.836776859504132	138.0	5.0	173.3	4.7482993197278915	68.0	484.0	7158.0
2341.0	68.92421619325587	1.4288247004403603					

5.1.3 Configuration File

The configuration file stores information about virtually all the parameters within ImaGene. This file is a simple XML file format which is easily saved and loaded from the ImaGene Parameter Settings Window. While the contents of this file are not important to most users, you should be aware that this file is required for batch processing. Advanced users integrating ImaGene into high throughput environments may also find the information useful.

Listed below is a sample ImaGene configuration file:

```
<?xml version="1.0" encoding="UTF-8"?>

<ImaGene_Parameters>
  <IGNORED_MEDIAN>true</IGNORED_MEDIAN>
  <PERIMETERTOAREA_FLAG>false</PERIMETERTOAREA_FLAG>
  <BACKGROUND_FLAG>true</BACKGROUND_FLAG>
  <POOR_FLAG>true</POOR_FLAG>
  <PERIMETER_FLAG>true</PERIMETER_FLAG>
  <PERIMETERTOAREA_THRESH>0.65</PERIMETERTOAREA_THRESH>
  <TOLERANCE>5.0</TOLERANCE>
  <STANDARD_LOW>5000.0</STANDARD_LOW>
  <OFFSET_FLAG>true</OFFSET_FLAG>
  <SHAPE_REGULARITY>true</SHAPE_REGULARITY>
  <CONTROL_TYPE_LOWTHRESH_0>45000.0</CONTROL_TYPE_LOWTHRESH_0>
  <NEGATIVE_FLAG>true</NEGATIVE_FLAG>
```

<BLANK_CV>5.0</BLANK_CV>
<IGNORED_AREA>true</IGNORED_AREA>
<POOR_THRESH>0.995</POOR_THRESH>
<CONTROL_TYPE_ID_0_0>Gene17</CONTROL_TYPE_ID_0_0>
<OFFSET_THRESH>60.0</OFFSET_THRESH>
<HOT_CV>3.0</HOT_CV>
<CONNECT_FLAG>false</CONNECT_FLAG>
<EMPTY_THRESH>0.3</EMPTY_THRESH>
<HOT_CHECK>false</HOT_CHECK>
<MEASURE_MEAN>true</MEASURE_MEAN>
<BACKGROUND_THRESH>0.9995</BACKGROUND_THRESH>
<MEASURE_STDEV>true</MEASURE_STDEV>
<CONTROL_TYPE_VARTHRESH_0>1.0</CONTROL_TYPE_VARTHRESH_0>
<MEASURE_AREA>true</MEASURE_AREA>
<PERIMETER_THRESH>25.0</PERIMETER_THRESH>
<STANDARD_CV>5.0</STANDARD_CV>
<AUTO_SEG>true</AUTO_SEG>

<CONTROL_TYPE_UPALERTTHRESH_0>0.0</CONTROL_TYPE_UPALERTTHRESH
_0>
<CONTROL_TYPE_IFLOWALERT_0>false</CONTROL_TYPE_IFLOWALERT_0>
<BLANK_LOW>0.0</BLANK_LOW>
<BACKGROUND_BUFFER>3.0</BACKGROUND_BUFFER>
<CONTROL_TYPE_NAME_0>hot</CONTROL_TYPE_NAME_0>
<EMPTY_FLAG>true</EMPTY_FLAG>
<MEASURE_MODE>true</MEASURE_MODE>
<ENFORCE_GRID>true</ENFORCE_GRID>
<OVERALL_FLAG>false</OVERALL_FLAG>
<MEASURE_TOTAL>true</MEASURE_TOTAL>
<SIGNAL_HIGH>1.0</SIGNAL_HIGH>
<PERIM_TO_AREA>true</PERIM_TO_AREA>
<SIGNAL_FLAG>false</SIGNAL_FLAG>
<BACKGROUND_WIDTH>3.0</BACKGROUND_WIDTH>
<BLANK_CHECK>false</BLANK_CHECK>
<ON_TOP>false</ON_TOP>
<CONTROL_TYPE_UPTHRESH_0>65000.0</CONTROL_TYPE_UPTHRESH_0>
<SIGNAL_THRESH>0.9995</SIGNAL_THRESH>
<FLEXIBILITY>0.0</FLEXIBILITY>
<FIND_NEGATIVES>false</FIND_NEGATIVES>
<SIGNAL_LOW>0.0</SIGNAL_LOW>
<STANDARD_HIGH>10000.0</STANDARD_HIGH>
<BACKGROUND_LOW>0.0</BACKGROUND_LOW>
<OFFSET>true</OFFSET>
<CONTROL_TYPE_IFUPALERT_0>false</CONTROL_TYPE_IFUPALERT_0>
<SPOT_AREA>true</SPOT_AREA>
<REGULARITY_THRESH>0.65</REGULARITY_THRESH>
<LOG_SCALE>false</LOG_SCALE>
<STANDARD_CHECK>false</STANDARD_CHECK>
<HOT_HIGH>100000.0</HOT_HIGH>
<REGULARITY_FLAG>true</REGULARITY_FLAG>
<CONTROL_TYPE_N>1</CONTROL_TYPE_N>
<MEASURE_MEDIAN>true</MEASURE_MEDIAN>

```
 <CONTROL_TYPE_IFCVALERT_0>false</CONTROL_TYPE_IFCVALERT_0>
 <CONTROL_TYPE_COLOR_0>-52429</CONTROL_TYPE_COLOR_0>
 <IGNORED_FLAG>true</IGNORED_FLAG>
<CONTROL_TYPE_LOWALERTTHRESH_0>0.0</CONTROL_TYPE_LOWALERTTHR
ESH_0>
 <OPEN_PERIMETER>true</OPEN_PERIMETER>
 <CONTROL_TYPE_ID_N_0>1</CONTROL_TYPE_ID_N_0>
 <BLANK_HIGH>1000.0</BLANK_HIGH>
 <BACKGROUND_HIGH>1.0</BACKGROUND_HIGH>
 <HOT_LOW>30000.0</HOT_LOW>
 <IGNORED_THRESH>25.0</IGNORED_THRESH>
</ImaGene_Parameters>
```

5.1.4 Batch File

The ImaGene batch file is an XML file which contains links to all the configuration files required to batch process images. The file is created, saved and loaded from within the Batch Editor Window. As with ImaGene configuration files, the contents are usually only relevant to advanced users and those who need to create a batch file from scratch. As ImaGene can also be launched from the command line, creating a batch manually and passing this information in as a command line argument provides a viable alternative than simply running the software from the Batch Editor.

Listed below is a sample batch file. Note that the information contained here matched the information required within the Batch Editor.

```
<?xml version="1.0" encoding="UTF-8"?>

<Batch>
 <Entry>
  <Image>C:\BioDiscovery\ImaGene\Samples\jk88&2.TIF</Image>
  <Template>C:\Documents and Settings\data\Desktop\Desktop.tpl</Template>
  <Configuration>X:\data\configuration.xml</Configuration>
  <Destination>C:\Documents and Settings\data\Desktop</Destination>
  <Channel>0</Channel>
  <ChannelName></ChannelName>
  <ImageId>0</ImageId>
 </Entry>
 <Entry>
  <Image>C:\BioDiscovery\ImaGene\Samples\1267_rTIF</Image>
  <Template>C:\Documents and Settings\data\Desktop\Desktop.tpl</Template>
  <Configuration>X:\data\configuration.xml</Configuration>
  <Destination>C:\Documents and Settings\data\Desktop</Destination>
  <Channel>0</Channel>
  <ChannelName></ChannelName>
  <ImageId>1</ImageId>
 </Entry>
 <Entry>
  <Image>C:\BioDiscovery\ImaGene\Samples\18998.TIF</Image>
```

<Template>C:\Documents and Settings\data\Desktop\Desktop.tpl</Template>
<Configuration>X:\data\configuration.xml</Configuration>
<Destination>C:\Documents and Settings\data\Desktop</Destination>
<Channel>0</Channel>
<ChannelName></ChannelName>
<ImageId>0</ImageId>
</Entry>
<Entry>
<Image>C:\BioDiscovery\ImaGene\Samples\198fdddty65.TIF</Image>
<Template>C:\Documents and Settings\data\Desktop\Desktop.tpl</Template>
<Configuration>X:\data\configuration.xml</Configuration>
<Destination>C:\Documents and Settings\data\Desktop</Destination>
<Channel>0</Channel>
<ChannelName></ChannelName>
<ImageId>1</ImageId>
</Entry>

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E-mail - support@biodiscovery.com
Phone - (310) 306-9310 (United States)
Fax - (310) 306-9101
Mail - 4640 Admiralty Way, Suite 710, Marina Del Rey, CA 90292, USA

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E-mail - info@biodiscovery.com
Phone - (310) 306-9310 (United States)
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contractor or subcontractor (at any tier), then the Government's rights in the Licensed Software and accompanying documentation shall be only as set forth in this Agreement; this is in accordance with 48 C.F.R. 227.7201 through 227.7202-4 (for Department of Defense (DoD) acquisitions) and with 48 C.F.R. 2.101 and 12.212 (for non-DoD acquisitions).

9. Governing Law. This Agreement will be governed by California law and controlling U.S. federal law. Neither the United Nations Convention on the International Sale of Goods nor the choice of law rules of any jurisdiction will apply. Any dispute relating to or arising out of this Agreement shall be resolved solely by an action filed in the Santa Clara County Superior Court or the United States District Court for the Northern District of California.

10. Severability. If any provision of this Agreement is held to be unenforceable, this Agreement will remain in effect with the provision omitted, unless omission of the provision would frustrate the intent of the parties, in which case this Agreement will immediately terminate.

11. Integration. This Agreement is the entire agreement between you and Sun relating to its subject matter. It supersedes all prior or contemporaneous oral or written communications, proposals, representations and warranties and prevails over any conflicting or additional terms of any quote, order, acknowledgment, or other communication between the parties relating to its subject matter during the term of this Agreement. No modification of this Agreement will be binding, unless in writing and signed by an authorized representative of each party.

12. Remedies. It is understood and agreed that, notwithstanding any other provision of this Agreement, your breach of the provisions of Section 3 of this Agreement will cause Sun irreparable damage for which recovery of money damages would be inadequate, and that Sun will therefore be entitled to seek timely injunctive relief to protect Sun's rights under this Agreement in addition to an and all remedies available at law.

13. Nonassignment. Neither party may assign or otherwise transfer any of its rights or obligations under this Agreement without the prior written consent of the other party, except that Sun may assign this Agreement to an affiliated company.

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